Fungal treatment of selected matured forages to improve their value for ruminant feeding

By

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A thesis submitted for the degree of Doctor of Philosophy

School of Natural and Environmental Sciences

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March, 2019

Abstract

Ruminant nutritionists have been faced with a lot of challenges in improving the digestibility of low quality feeds that are available for ruminants in the dry season in most tropical regions. In view of this, they are interested in finding low cost approaches that are not hazardous to the animals, people and the environment. The use of biological methods (i.e. microbes) has been found to be capable of breaking down the structural components of these feeds such that the animals can access the energy components of the feeds. Among these microbes fungi have been found to be more efficient in degrading the structural components due to their enzymatic activity. Therefore, a series of experiments was carried out to evaluate the effectiveness of both aerobic and anaerobic fungi in improving some of the most available and abundant forages in Nigeria alongside two commonly used British forages.

The aerobic experiment using CR (*C. rivulosus*) and PO (*P. ostreatus*) was conducted to improve the nutritive value of selected matured forages (*A. gayanus*, *B. decumbens*, and *T. aestivum*) along with *L. perenne* that was used as a benchmark by degrading lignin. However, there are several reports where fungal treatment does not improve the nutritive value of forages. This research, therefore, investigated one possible reason that may have caused this, which is the removal of solubles caused by improper handling of pre-treated forages on the farm using PFF (pump filtered method; representing the use of heavy stones on sacks) and FFF (free filtered method; representing the use of porous sacks). These 2 methods were applied on the selected forages after pre-treatment was carried out using two substrate-liquid ratios (1:3 & 1:5) and incubated at 20^0C (CR) and 25^0C (PO) for 14 days and 28 days respectively. The use of both methods resulted in soluble losses in the fungal pre-treated forages but more loss was obtained through the PFF method. The PFF method did negatively influence the ability of the fungi to improve the chemical composition of the pre-treated forages in comparison with the FFF method. Also, the PFF method did not improve the *in vitro* nutrient degradability and *in vitro* fermentation parameters of the fungal-pre-treated forages.. Farmers should endeavour to ensure that losses of solubles are minimal and consistent by using containers/bags/sacks that will not allow the escape of solubles during pre-treatment, and also ensure that no pressure is exerted on the pre-treated forages. The use of growth conditions favouring excessive growth and ligninolytic function by the fungi on the substrate should be avoided when implementing the upgrading of forages on the farm, as this leads to the release of more solubles that might be subsequently utilised by the fungi or lost through improper handling.

The anaerobic experiment using (*Neocallimastix* and *Opinomyces)* was conducted to improve the nutrient utilisation of the selected matured forages when used as feed additives. These fungi were used as silage inoculants for the selected matured forages over 2 inoculation times (i.e. 14 and 28 days). The supplementation of these two anaerobic rumen fungi as silage inoculants improved the silage quality of the forages. This improvement was reflected in the reduced pH, increased CP content, decreased fibre content, increased metabolite contents especially by *Orpninomyces* sp., and increased TAN contents especially by *Neocallimastix* sp. This improvement was complemented with a minimal nutrient loss. *Orpinomyces* sp. had a greater preference in degrading fibre in the temperate forage silages, i.e. *L. perenne* silage and *T. aestivum* silage, while *Neocallimastix* sp. had a greater preference in degrading fibre in the tropical forage silages, i.e. *A. gayanus* silage and *B. decumbens* silage. Although the silage was improved, the fibre content was still high, but there is the possibility that the chemical bonds that exist between lignin and other polysaccharides might have been loosened during ensiling. These ensiled forages, therefore, require further degradation by rumen microbes for the utilisation of nutrients by ruminant animals.

Declaration

I confirm that the work undertaken and written in this thesis is my own work that it has not been submitted in any previous degree application. All quoted materials are clearly distinguished by citation marks and source of references are acknowledged.

The articles published in conference proceedings from the thesis are listed below:

Bolaji, O. J., Chaudhry, A. S., and Dolfing, J. (2015). Chemical evaluation of selected Nigerian matured forages for their use in ruminant diets. Proceeding of the British Society of Animal Science, S. Athanasiadou, Chaudhry, A. S., Chikunya, S., Denwood, M., Flockhart, J., Flynn, R., Gibbons, J., Houdijk, J. G. M., Kenny, D. A., Mather, A., Mayes, R. W., McNeilly, T., Nash, D. M., Richardson, R. I., Rooke, J. A., Rose, M. T., Steele, M. A., Rymer, C., Wall, E., Wolf, B. T. and Wylie, A. R. G. (Eds.) 112. Published by Cambridge University Press.

Bolaji, O. J., Chaudhry, A. S., and Dolfing, J. (2016). *In vitro* degradability of selected Nigerian forages for their use in ruminant diets. 67th Annual Meeting of the European Federation of Animal Science (EAAP), E., Strandberg, Van Duinkerken, G., Spoolder, H., Sauerwein, H., Tichit, M., Klopčič, M., Conington, J., Lauridsen, C., Santos, A., and Pollott, G. 410. Published by Wageningen Academic.

Acknowledgement

I give all glory, honour and adoration to the Almighty God, the author of wisdom, knowledge and understanding for the grace He has bestowed upon me to be able to complete this programme, without Him wouldn't have been able to accomplish it.

My profound gratitude goes to my major supervisor, Dr. Abdul S. Chaudhry for his moral, academic, and physical support, your kind way of corrections has made me a better researcher today, you have been a wonderful supervisor to me throughout the years of my study and I will never forget you. My sincere appreciation goes to my second supervisor, Jan Dolfing for his fatherly and academic support and words of encouragement that has held me on up till this day of completion of thesis. It has been wonderful and a great privilege having you both as my supervisors.

My appreciation goes to Tertiary Education Trust Fund (TETFund), Nigeria for the financial support granted for three years of the PhD programme. I am also indebted to my employer Federal University of Agriculture, Abeokuta (FUNAAB) for giving me the opportunity to be able to study overseas. I am also grateful to some member of the Department of Pasture and Range Management (Prof J.A. Olanite, Prof M. Arigbede, Dr. (Mrs.) V.O.A. Ojo, Prof Onifade, and Prof. (Mrs.) A. Jolaosho), staff in the college (Dr. R. Shobayo, Dr. O.M.O Idowu, Dr. A. Adelusi), my academic mentor (Prof David Adejare Agboola) and many people whose names may not all be enumerated for their moral and academic support, you have all been wonderful.

My profound gratitude goes to the technical staff in Cassie building (David Race, Sarah, Amy, and Prince Harry) and in Agriculture building (Peter Shotton, Wendy Ball, Fiona Maclachlan, and Roy Lamb) both at Newcastle University for your assistance and support in carrying out laboratory analyses, really appreciate it. Thanks to all my PhD colleagues and besties (Tony Aiyelari, Oak, Siti, Essien, Erika, Nur, Carl Samuel, and Julija), and others who in one way or another had shared their support during the programme, you all have been fantastic.

I will like to express my deep appreciation to my husband (Idris Babatunde Idowu) who has been there for my during the programme by rendering both physical, emotional, and financial support, you have indeed proved yourself to be a loving, caring and wonderful husband. Also to my daughter (Inioluwa Elizabeth Idowu) for your understanding and patience throughout the programme. Lastly will like to appreciate my father (Engineeer Adisa Bolaji), mother (Mrs Esther Bolaji), brother (Engineer Segun Bolaji), sisters (Kemi, Ope, Tolu, and Korede) and my in-laws for their prayers and financial support that has helped in completing this study.

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Chapter 1

General Introduction and Literature Review

1.1 General Introduction

During dry season, ruminants in tropical countries such as Nigeria depend on low quality feeds including crop residues such as cereal straws, corn stover, tree legumes, and grasses etc.. This is contrary to the diets that are available to ruminants in temperate countries (Jackson, 1981).These feeds are usually high in lignocellulose (i.e. hemicellulose, cellulose and lignin), and they require further upgrade and/or improvement before they can be properly utilised for ruminant production. Among these feeds, crop residues have received much attention for their improvement as a useful nutrient resource for ruminants (Okano *et al.*, 2005; Arora and Sharma, 2009a; Akinfemi, 2010b; Kaur *et al.*, 2010; Sharma and Arora, 2010a; Akinfemi and Ladipo, 2011; Arora and Sharma, 2011; Arora *et al.*, 2011; Shrivastava *et al.*, 2012; Kumar and Sharma, 2017; Khota *et al.*, 2018; Nayan *et al.*, 2018). Moreover, tree legumes are generally used as protein supplements as they contain high protein and carbohydrate contents (Van Eys *et al.*, 1986; Ondiek *et al.*, 1999; Camero and Franco, 2001; Aye and Adegun, 2013; Dubeux Jr *et al.*, 2015; Vandermeulen *et al.*, 2018) . On the other hand, the standing grasses have received little or no attention for their upgrade, even though they have higher nutritive value than cereal straws. Also, they are of immense benefit in that they provide Vitamin A and E for ruminant animals, which are very important in maintaining a balanced immune system and health condition (Lonn and Yusuf, 1997; Melanson *et al.*, 2003; Daley *et al.*, 2010). However, as season and maturity tend to limit the importance of green fodder to animals, there is the need to investigate the nutritional improvement of these green forages. In Nigeria, green forages such as *Cynodon* spp, *Eleusine coracana, Brachiaria* spp, *Andropogon* spp, *Pennisetum* spp, *Panicum* spp etc. have been found to be promising as possible improved dry season feeds due to their persistence, hardiness and heat tolerance (Adenova, 1985; An and Reynolds, 1989).

Several studies have been carried out towards maintaining and improving the nutritional values of these grasses by conserving them in the form of hay and silage (Muck and Shinners, 2001); supplementing them when fed with urea and ammonia etc. (Dolberg, 1992; Oji *et al.*, 2007; Wanapat *et al.*, 2009); and reducing the particle size by milling, heating and irradiating. These methods give a short-term solution towards improving the nutritive value of grasses due to the high cost of chemicals, requirement for high energy and pressure (steam and electricity), environmental hazards and lack of consumer preference for chemicals etc. (Keller *et al.*, 2003; Silverstein *et al.*, 2007; Lee *et al.*, 2009; Kumar and Sharma, 2017). Given this, they are not yet

adequately utilized for breaking down and/or degrading the lignified cell wall of these grasses and thus improving their digestibility by ruminant animals.

In achieving better degradation of lignocellulose, fungi have been identified as a reliable resource due to their various characteristics. These characteristics include their preference for diets rich in lignocelluloses, i.e. stemmy or stalky forages (Grenet *et al.*, 1989), which tend to increase fungal population and abundance (Grenet and Barry, 1988; Bauchop, 1989; Edwards *et al.*, 2008); possession of motile zoospores that attach and colonise substrates with lignified or thick cell walls (Edwards *et al.*, 2008) and possession of rhizoids or hyphae that penetrate complex intra-cellular plant tissues that act as barriers (Gordon and Phillips, 1998; Abdel-Hamid *et al.*, 2013; Mahesh and Mohini, 2013; Abrão *et al.*, 2014; Leis *et al.*, 2014). These physical actions of fungi can disrupt and open up the internal plant structures (Akin, 1989) for further breakdown by other rumen microbes and enzymes (Dagar *et al.*, 2011). These enzymes could be either hydrolytic, such as cellulases, hemicellulases, proteases, esterases, chitinases (Orpin and Joblin, 1997; Dey *et al.*, 2004; Puniya *et al.*, 2015), or ligninolytic like lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and versatile peroxidase (Higuchi, 2004; Dashtban *et al.*, 2009; Wong, 2009). These enzymes loosen chemical bonds (Dashtban *et al.*, 2009) and linkages that exist between lignin and polysaccharide complexes (Borneman *et al.*, 1992; Paul *et al.*, 2003). These characteristics make fungi capable of increasing the nutritive value and digestibility of these low-quality forages and manipulating the rumen ecosystem for better utilisation giving improved ruminant production. Two groups of fungi, i.e. wood decaying and anaerobic, have the potential for improving low-quality feeds for better ruminant production. However, they vary in the way they achieve this purpose.

Anaerobic rumen fungi (ARF) are used as additives in the form of directly fed microbes (DFM) or probiotics when lignified plant tissues are made available to ruminants. These additives are used in manipulating the rumen microbial ecosystem by increasing the number and activity of cellulolytic microbes in the rumen (Dayananda *et al.*, 2007; Tripathi *et al.*, 2007b; Tripathi *et al.*, 2007a) and thus altering the fermentation pathway which leads to an increased nutrient utilization (Thareja *et al.*, 2006; Sehgal *et al.*, 2008). Moreover, variable increases in voluntary feed intake (Theodorou *et al.*, 1990; Ha *et al.*, 1994; McAllister *et al.*, 1994; Gordon and Phillips, 1998; Dey *et al.*, 2004; Paul *et al.*, 2004), nutrient digestibility of especially fibre (Arambel *et al.*, 1987; Lee *et al.*, 2000a), growth rate and feed efficiency (Dey *et al.*, 2004; Thareja *et al.*, 2006), nitrogen retention, production efficiency and animal performance (Lee *et al.*, 2000a) and milk production (Saxena *et al.*, 2010) have also been reported. Also, the use of anaerobic fungi in ruminant feeds seems to be a safe method in the food supply chain to

consumers (Puniya *et al.*, 2015) because they have no obvious residual or side effects when used in animal diets. This might be because they exist naturally in the rumen environment (Dagar *et al.*, 2011) and they show a symbiotic relationship with the animals (da Silva *et al.*, 2017). Among these ARF, much work has been done using *Neocallimastix* sp. (Sehgal *et al.*, 2008), *Orpinomyces* sp. (Lee *et al.*, 2000a; Mani Kumar *et al.*, 2002; Manikumar *et al.*, 2003; Dey *et al.*, 2004), *Piromyces* sp. (Paul *et al.*, 2004; Paul *et al.*, 2006; Tripathi *et al.*, 2007a) etc., when these fungi were isolated and co- cultured with other rumen microbes through either *in vivo, in sacco* and *in vitro* approaches (Grenet and Barry, 1988; Bernalier *et al.*, 1991; Weimer, 1998; Lee *et al.*, 2000a; Paul *et al.*, 2004; Thareja *et al.*, 2006; Kim *et al.*, 2008; Nagpal *et al.*, 2011) or by utilising liquid media containing different sources of fermentable carbon (Gordon and Phillips, 1989). It has been identified that, in a co-culture microbial environment, a decrease in cellulolysis did occur as compared to a mono-culture microbial environment (Lee *et al.*, 2000a) due to the presence of protozoa that inhibited the cell wall degradation rate as well as the activity of cellulolytic bacterial and fungal fraction. In most cases, the substrates being used in the co-culture environment were cereal straws and not low-quality grasses. Also, there is little information on the use of isolated ARF for pre-treatment of substrates before introducing the feed into the rumen environment, through either an *in vivo* or *in vitro* approach, in order to achieve double benefits of the ARF treatments. In achieving better upgrade of low quality forages by these anaerobic fungi, there is the need to investigate the way they upgrade the forages, as they seem to vary in their hydrolytic function, even in the presence of the same substrate.

White rot fungi, among the wood decaying fungi, do improve pre-treated low quality forages by degrading lignin effectively without much attack on cellulose and hemicelluloses (Zadražil and Brunnert, 1982a; Blanchette, 1995; Keller *et al.*, 2003; Guillén *et al.*, 2005) that serve as major nutrients for animals (Haug, 1993). Lignin is a major component of the plant cell wall that usually limits digestibility by rumen microbes due to its recalcitrant nature and the complex polymer of phenyl propane units that are usually cross-linked to each other with a variety of different chemical bonds. Among the white rot fungi, researchers have been able to identify some white rot fungi that selectively degrade lignin such as *Phlebia* species (Fackler *et al.*, 2006; Arora and Sharma, 2009a), *Ceriporiopsis subvermispora* (Guerra *et al.*, 2004; Fernandez-Fueyo *et al.*, 2012), *Ceriporiopsis rivulosus* (*Physisporinus rivulosus*) (Hildén *et al.*, 2007), *Dichomitus squalens* (Bak *et al.*, 2010), and *Pleurotus* spp (Vane *et al.*, 2001; Assi and King, 2007; Fazaeli, 2007). Selective white rot fungi have the capability of improving the nutritive quality of various agro-residues through, e.g. increased nutrient digestibility (Arora *et al.*, 2011;

Shrivastava *et al.*, 2012; Tuyen *et al.*, 2012; Tuyen *et al.*, 2013) and antioxidant ability (Pouteau *et al.*, 2003; Sharma *et al.*, 2010) but reduced methanogenic potential.

Edible mushroom producing fungi and their mycelium are regarded as safe for ruminant feeding (GRAS, i.e. generally recognised as safe, status; an American food and drug administration classification) as no case of fungal pathogenicity have been recorded toward animal species feeding on the treated substrates and at the long run the end users i.e. human (Sharma and Arora, 2015). Also, some fungi such as *C. rivulosus* and *C. subvermispora* are not mushroom producing fungi. In fact they are selective white rot fungi and their use has not been ascertained as safe for feeding, although some researchers who have used them in feeding animals (Okano *et al.*, 2009) did not identify any harmful effect on animals when used in a compound diet over a short period. However, there is the need to continually investigate fungal fermented substrates for mycotoxins / aflatoxin when new treatment is proposed as Sharma *et al*. (2012)was able to identify the presence of several mycotoxins in different fungal fermented wheat straw, although their presence was lower than 20ppb which is regarded as the permissible level in immature animals diet and poultry.

Researchers have reported that some aerobic and anaerobic fungi were capable of solubilising toxic metabolites and or phenolic compounds in waste water (Paszczynski and Crawford, 1995) and forage substrates (Makkar *et al.*, 1994; Paul *et al.*, 2006; Puniya *et al.*, 2014). This indicates that fungal treatment might be used as an option in improving potential ruminant feeds that contain high tannin contents for their application in ruminant diets. However, there is still a need to investigate the effect of different fungi on the nutritive value of some low-quality forages, as they exhibit different enzymatic functions, even in the presence of the same substrate. Surprisingly, most of these researchers have been using cereal straws as their major lignocellulosic substrate, whereas those green grasses that stay longer into the dry season in some tropical countries have received little or no attention. Therefore, the following literature review examines the past research and future potential in using various procedures to improve the nutritive value of various forages for ruminant feeding.

1.2 Literature Review

1.2.1 *Available Ruminant feed as influenced by the region*

Ruminants are animals (e.g. cattle, sheep, goats, camels, and wild ruminants like, gazelles, giraffes, and buffalos) that depend on green forage as their major source of feed (Brum *et al.*, 2008). Feed is a major factor in the production of ruminant animals as it contributes about 70% of the cost of production. However, the season has been the principal factor influencing the

available feed in each region (i.e. temperate and tropical regions). In temperate regions, the seasons are usually not characterized by higher temperature, higher precipitation and higher sunlight, which are common features of tropical regions. These extreme weather conditions in the tropical regions has led to rapid growth rate and early maturity of the forages found in these regions. The available feeds in temperate regions are mostly of high quality, such as hay, silage, concentrate and green forages. These feeds are usually characterized by high protein content (Buxton, 1996; Wilkins, 2000; Minson, 2012), low insoluble carbohydrates and fibre (Wilson, 1994), high feed intake due to high palatability and consequently fast passage rate through the rumen (Van Soest, 1994; Fisher, 2002). Maturity has little impact on the temperate forages, thus producing a feed (e.g. hay and silage) of higher quality, and season in most cases has little effect on the dry matter content (Adesogan *et al.*, 2006).The time spent on grazing by an animal is reduced with the temperate forages.

On the other hand, in the tropical regions during the dry season**,** the available feeds are usually low-quality feeds, such as crop residues, agro-industrial by-products, kitchen waste, and low quality green forages (Bakrie *et al.*, 1996; Leng, 1996; Mahesh and Mohini, 2013). The tropical green forages are characterized by low energy and crude protein but high fibre, i.e. energy and protein are not available in the right quantity (Wilkins, 2000; Minson, 2012), and low digestibility which is usually associated with lower feed intake. Season tends to increase the dry matter content (Preston, 1982; Yayneshet *et al.*, 2009). Much grazing time is exerted while ingesting the forage (Van Saun, 2006) and improvement of forages is directed towards increasing the DM digestibility of the forage species. For instance, improved Napier grass (a hybrid produced from *Pennisetum purpureum* and *Pennisetum glaucum*) recorded a higher digestibility than un-improved forage at early stage of growth (Minson, 1971).

1.2.2 *Available Feed Resources in Nigeria*

The feed resources available for either small or sizeable ruminant production are mainly low-quality forages (LQF) such as crop residues and agro –industrial by-products, perennial food crops (e.g. sugarcane, bananas, roots and tubers, kitchen waste), natural pastures (pastures that grow on an area of land without being cultivated) and green forages. These LQF are usually characterized by lesser digestibility (less than 55%) and lower crude protein (less than 70g/kg) (Sampaio *et al.*, 2009). They usually form the primary feed for large ruminants in both tropical and sub-tropical countries throughout the year (Jackson, 1981). The crop residues and agro-industrial by-products represent a major feed source in most tropical countries, where lands are used for the production of human foods. Several residues of crops such as maize, wheat, rice, paddy and pulses are used for animal feeding. However, maize produces the highest amount of

residues worldwide than all the other crops listed. Some other sources of crop residues include sorghum stover, sugarcane tops and leaves, oil plant stovers and foliage, barley straws, roots and tubers (Preston, 1986; Owen and Jayasuriya, 1989; Devendra, 2009). Supplementation of these crop residues with by-pass protein and nutrient blocks (i.e. urea molasses) increases their palatability, thus facilitating the rate of feed passage in the rumen. On the other hand, chemical pre-treatments with urea/ammonia help in increasing the microbial breakdown of plant cell walls by rumen microbes, therefore enhancing digestibility (Liu *et al.*, 2005; Silverstein *et al.*, 2007; Kumar and Sharma, 2017).

Food crops such as sugarcane, cassava, and banana etc., which are usually grown for human consumption, are now used for feeding ruminant animals, especially during the dry season. Higher dry matter/ha and nutrients have been found to be supplied by these food crops, since they are not influenced by stage of growth or maturity. This makes them more flexible than pastures.

Green fodder, especially grasses with high nutritive value, are of immense benefit, especially in the provision of essential minerals, ammonia, peptides/amino acids and vitamin A for ruminant animals. Vitamin A is essential in maintaining a balanced immune system and health condition, and it improves the lactating capacity of animals. Other benefits of the green fodder include: prevention of respiratory disorders; prevention of digestive disorders (such as diarrhoea, absorption of nutrients, etc.); prevention of urinary tract disorders (such as stones in kidneys, ureter and bladder); provision of minerals, crude protein, total digestible nutrients, dry matter and carotene etc. Also, the inclusion of green forages in the straw-based diets of ruminants increases the digestibility (Ocen, 1992; Jung and Allen, 1995; Brum *et al.*, 2008; Elejalde *et al.*, 2010) and reduces the demand for additional nutrients by the animals (Leng, 1991).

However, seasonal management practices and maturity tend to limit the above listed importance of green fodder to animals and so there is a great need to investigate the potential of nutritionally improving these green forages. On natural pastures, especially on the open derived savannah, some grasses such as *Imperata cylindrica*, *Hyparrhenia* spp., *Andropogon gayanus, Brachiaria* spp., *Panicum maximum*, and *Pennisetum purpureum* etc. (Adenova, 1985; An and Reynolds, 1989) are more predominant and are readily available irrespective of the season. This is because, during the wet season, these grasses tend to grow rapidly to the extent that they are under grazed and become lignified (Leng, 1996) during a later period of the wet season, then further become more lignified as the dry season persists.

This lignification makes nutrients in these green forages, especially grasses, inaccessible for the animals and therefore leads to low production during the dry season (Leng, 1990; Odenyo *et al.*, 1997; Shelton, 2004). Even in the later period of the wet season, the available nutrients in these grasses can only sustain the animals for their maintenance requirement (Smith, 1989). During the dry season the average crude protein content tends to fall from 10% to 2%, the ash content reduces from 6% to 3-4% while the crude fibre content and acid detergent fibre contents rise from 32% and 43% to 50% and 60% respectively. There is the need to supplement these grasses with browse plants (fodder trees and shrubs) and crop residues to obtain an optimum production.

1.2.3 *The plant cell wall components and factors limiting forage digestion*

Plant cells are well-organised structures formed at the meristems. They develop into cell types which are then grouped into three tissues: dermal (i.e. the epidermal cells that cover the outer parts of tree plants or plant cell walls); ground (these make up the majority of primary body of plants such as the parenchyma, sclerenchyma and collenchyma cells); and vascular, the connecting tissues (phloem, cambium, xylem and parenchyma cells that aid in the transportation of water, food, minerals and hormones within the forage plant). Plant cell wall is made up of a structural material called lignocellulose; a major component of plant biomass.

Lignocellulose is made up of three constituents, i.e. cellulose, hemi-cellulose and lignin (Lee, 1997; Sjostrom, 2013). These components are covalently and non- covalently bonded to form a complex structure that is very difficult to dissociate. Cellulose occupies a more significant portion of the substrate, followed by hemicellulose and lastly by lignin (Baldrian and Valášková, 2008). Cellulose is a crystalline structure that is compacted to form micro-fibres. These micro fibres are well packed and found between lignin layers which protect it and makes it difficult for possible breakdown by several methods of hydrolysis (i.e. physical, chemical or biological processes) (Leonowicz *et al.*, 1999).

1.2.3.1 *Cellulose*

A polysaccharide is composed of β-1, 4-linked D-glucose units with several polymers having hydroxyl residues at C_2, C_3 and C_6 atoms. These polymers are linked to one another with hydrogen bonds and interactions involving Vander Waals to form long chains (Sánchez, 2009). Cellulose occupies about 30–50% dry weight of lignocellulose. The bonding of hydroxyl residues to oxygen atoms on the close-by chain, leads to the production of micro fibrils. Cellulose chains vary in length due to the source of cellulose, having glucose units ranging from 2000 to 12000. Rotation of a glucose unit has to be relative to its two close-by glucose units at 180^0, to form disaccharide cellobiose (i.e. a glucose polymer) (Zhang and Lynd, 2004).

Cellulose which is highly crystalline in nature exists in several types of cellulose allomorphs called cellulose I, II, III, and IV. Most wood and higher plants have the cellulose in the form of the cellulose I allomorph, which is usually surrounded by lignin and hemicellulose (Howell *et al.*, 2011). The crystallinity of cellulose in plant cells varies depending on the cellulose source. However, the crystallinity exists at various regions in a plant cell which are separated by amorphous regions. Cellulose I, a naturally occurring variant, is made up of crystalline forms of triclinic (1α) and monoclinic (1β) mixtures (Atalla and VanderHart, 1984). Cellulose II is a type of cellulose obtained from cellulose fibre regeneration. Cellulose III is formed from liquid ammonia treatment of cellulose I or II. Cellulose IV is formed from cellulose III treatment with heat (Zugenmaier, 2001). Cellulose can be converted to useful products by the action of cellulose degrading enzymes.

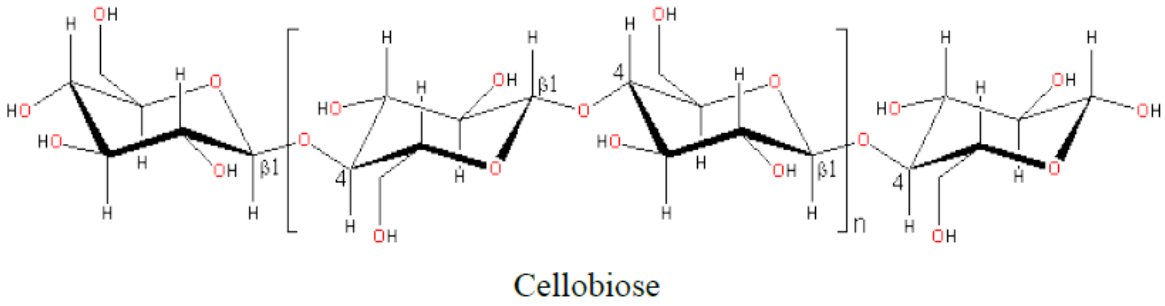


Figure 1 Schematic representation of cellulose backbone. Adapted from Brown *et al.* (1996)

1.2.3.2 *Hemi-celluloses*

This is a heterogeneous polysaccharide that is very complex. It consists of D- galacturonic, D-4-0-methyl-glucuronic, D-xylose, D- arabinose, D- glucuronic acid, D-mannose, D- galactose, and other acetyl molecules that are linked by ester bonds, etc. (Pérez *et al.*, 2002). Its structural complexity makes it prone to requiring more enzymes for its degradation, although not crystalline in nature like cellulose. This non-crystallinity exposes it to efficient enzymatic breakdown (Saha, 2003). Hemicellulose occurs predominantly more in the secondary plant cell wall than in the primary cell wall, and it is found closely linked to cellulose. The fraction of hemicellulose in the plant varies, e.g. glucomannan, and glucuronoxylan are the hemicellulose fractions of grasses or hardwoods and softwoods (Jeffries, 1994). Hemicellulose is made up of different sugars linked to each other , by mostly β-1, 4-glycosidic bonds and in a few cases by β-1,3glycosidic bonds (Pérez *et al.*, 2002). Xylan is another hemicellulose that is more abundant and structurally more complex than any other hemicellulose (Mazeau *et al.*, 2005). Hemicellulose consists of major sugars (i.e. arabinose, glucose, xylose, galactose, mannose and

glucuronic acid). The structure of hemicellulose consists of a single sugar type on a long linear chain while acetate and other sugars are found on side branched chains (Duff and Murray, 1996). Most plants have β (1-4)-D-xylopyranan as the sugar constituent of the extended linear chain while α-D-glucopyranosyl uronic acid units with 4-0-methylether and α-L-arabinofuranosyl are the components of the branched side chains (Mazeau *et al.*, 2005). The constituents of hemicellulose vary with plant species and between various parts (i.e. stem, leaves and roots) of a particular plant (Pérez *et al.*, 2002).

1.2.3.3 *Lignin*

This is made up of complex units of phenylpropane (i.e. a polymer), where the units are cross-linked by chemical bonds of a different variety. This complex nature makes it resistant to microbial degradation and microbial enzymatic penetration, therefore physically restricting the bioavailability of other cell wall components that would have served as important nutrients to animals (Haug, 1993). Lignin is a complex plant cell wall component located mostly in plant xylem, and its complexity is due to the presence of the three-dimensional aromatic heteropolymer (Donaldson, 2001). Lignin is made up of three alcohol constituents namely: p-hydroxy phenyl propanol (p-coumaryl alcohol), syringyl alcohol (sinapyl alcohol), and guaiacyl propanol (coniferyl alcohol). The polymerisation of these alcohols leads to the synthesis of the three-dimensional polymers of lignin (Buranov and Mazza, 2008). Within the lignin polymerization, several linkages (i.e. inter-phenyl propanol linkages) exist. These linkages are more than ten, and they include the β-1, β-5, α-0-4, β-β, 5-5, β-0-4 and 4-0-5 linkages with β-0-4 being the most occurring linkage between units, occupying about 50% of the units (Lee, 1997). Peroxidase biosynthesis occurs mainly from the couplings of radicals (Buranov and Mazza, 2008). Secondary reactions do take place during polymerization, and this leads to cross-linking of the fractions of hemicellulose and lignin. The lignin fraction varies with plants, e.g. syringyl and guaiacol are the lignin components in hardwood while coniferyl alcohol is found in softwood (Jeffries, 1994; Pérez *et al.*, 2002).

Lignin in plants plays several roles such as it provides plant cell wall with structural support, protects the plants from oxidative stress and microbial attack and improves conduction in cell saps (Boudet, 2000). Lignification occurs when lignin fills up inter-lamella cavities by occupying the carbohydrate matrix, therefore bonding with carbohydrates that are non-cellulosic. The process of lignification starts by spreading from the middle lamella to the secondary wall and lastly it gets to the lumen (Donaldson, 1991). Lignification of the middle lamella is complete on the onset of lignification of the secondary wall, while secondary wall becomes lignified when the wall is finally constructed (Donaldson, 1991).

1.2.3.4 *Interactions existing between lignin- carbohydrate and cellulose-hemicellulose*

Plant cell walls are very essential in bringing about structural stability and nutrient transport within the plants. Various interactions or complexes (lignin-carbohydrate and hemicellulose-cellulose) formed within the plants greatly influence plant rigidity, plant structure and composition. The lignin-carbohydrate complexes formed in softwoods are unique, due to the different type of carbohydrates (e.g. arabino-4-0-methyl glucuronoxylan, arabinogalactan and galactomannan) that are linked to lignin (Azuma *et al.*, 1981) than the type of carbohydrate (arabino-4-0-methyl glucuronoxylan and 4-0-methylglucuronoxylan) linked to lignin in hardwoods and grasses.

Several linkages such as hydrogen bonds, α-ester and α-ether are formed between the lignin carbon complexes, but hydrogen bonds predominate (Zhang *et al.*, 2011). The structural properties and biosynthetic processes of lignocellulosic substrates are dictated by the types of linkages (i.e. Vander-Waals or hydrogen bonds) that exist between hemicellulose and cellulose complexes (Atalla *et al.*, 1993). These also prevent flocculation by binding to the micro-fibrils of cellulose through the formation of the acid coating (Dammström *et al.*, 2008). The existence of the lignin in the plant cell wall is not in a pure fraction because it is usually found linked to hemicellulose and cellulose, thus forming several complexes. The influence of lignin in various interactions differs. It has been observed influencing lignin-carbohydrate complexes in the secondary cell wall but does not affect the hemicellulose-cellulose complexes (Zhang *et al.*, 2011).

During the construction of plant cell wall, the structure of lignin is affected by alteration of the structure of the cell wall caused by genetic modification of the pathway involved in the biosynthesis of hemicellulose (Uhlin *et al.*, 1995). In softwoods, the hemicellulose xyloglucans are the most occurring, occupying a larger fraction of contained hemicellulose. Three macromolecular domains are used in the organisation of xyloglucans. One of the domains, regarded as the major domain, is closely linked to the cellulose - micro fibrils surface, while the remaining two domains exist within other cellulose micro fibrils in a cross- linked way (Chambat *et al.*, 2005). Another hemicellulose in softwoods, i.e. glucomannan, is closely linked to cellulose, while in similar way xylan is also linked to cellulose (Dammström *et al.*, 2008). The bond formed between hemicelluloses and celluloses varies, e.g. glucomannans bonds more strongly with higher affinity than xylans (Åkerholm and Salmén, 2001).

1.2.4 *Upgrading the LQF by pre-treatment to improve their nutritive value and digestibility.*

Highly lignocellulosic substrates such as cereal straws, agricultural by-products, crop residues and highly lignified pastures tend to supply energy for ruminants, but these are not usually

available for good animal utilization due to a high level of lignin (Jung, 1989). To obtain the best use of the energy for animal production, there is a need to expose the polysaccharide (i.e. cellulose and hemicellulose) sites for further enzymatic hydrolysis. The accessibility of these polysaccharides makes it possible for more energy in the form of sugars to be released. Several methods such as physical, chemical, physico-chemical and biological have recently been developed and used in pre-treating lignocellulosic substrates in order to facilitate the release of these polysaccharides for animal use and other industrial purposes (Alvira *et al.*, 2010). The effectiveness, efficiency and process of these methods have been discussed extensively by several authors (Sarkar *et al.*, 2012; Chaturvedi and Verma, 2013; Kumar and Sharma, 2017). Pre-treatment is carried out for the following purposes (Sanchez and Cardona, 2008): to reduce the crystallinity of cellulose to a form that can undergo better hydrolysis; to ensure that the contained hemicelluloses in the substrate are properly utilised; and to ensure that the lignin is removed or broken down. Also an ideal pre-treatment method should facilitate higher hydrolysis of the substrate after pre-treatment to release the sugars, require low energy, limit the production of inhibitory compounds, reduce capital and operational cost, avoid substrate size reduction and reduce polysaccharide loss (Gupta and Verma, 2015)

1.2.5 *Physical / mechanical pre-treatment*

This involves reducing the lignocellulosic substrates from larger to smaller particles to disrupt the crystallinity and amorphotic matrix of cellulose, create sites for further enzymatic hydrolysis and aid in increasing sugar yields, thus increasing intake and digestibility of such substrates. The physical method includes mechanical extrusion (i.e. heating lignocellulosic biomass under shear mixing at higher temperature ($>300^0$C)); pyrolysis (i.e. degradation of lignocellulosic biomass at high temperature ($500 – 800^0$C) without the use of oxidizing agent); comminution (i.e. grinding and milling (sizes between 0.2-2mm) as well as chipping (10-30mm) of lignocellulose); ultrasound; microwave and pulse electric field (Agbor *et al.*, 2011; Chaturvedi and Verma, 2013; Kumar and Sharma, 2017).

The physical pre-treatment method alone can disrupt cellulose structure but can't solubilise lignin, thus yielding a lesser amount of sugars compared to chemical pre-treatment. These physical methods have some limitations in that they involve a high amount of energy and the use of machines, thus making it difficult to scale up and expensive for use for industrial purposes, as depreciation of equipment is very high (Sánchez, 2009; Zhu and Pan, 2010; Sharma *et al.*, 2017). Also, the reduction in size of substrates to fine particle size (milling) below 0.4 mm in ruminant feed has been discovered to have no positive effect on hydrolysis rate and yield (Chang *et al.*, 1997).This can lead to reduction in the time spent in the chewing,

reduction in the ruminal pH, reduction in acetate: propionate ratio, reduction in fat content (Jaster and Murphy, 1983; Grant *et al.*, 1990), depression of ruminal cell wall component digestion rate therefore reducing digestion (Hogan and Weston, 1967), although it might increase feed intake. However when processed in the form of pellets and/or available in a total mixed ration, or used as supplements with other feedstuffs in animal feeds, it lead to higher feed intake and digestibility (Wondra *et al.*, 1995; Yang *et al.*, 2001; Krause *et al.*, 2002).

1.2.6 *Physico-chemical pretreatment*

This involves auto-hydrolysis (steam explosion), liquid hot water, wet oxidation, SPORL (sulphite pre-treatment to overcome recalcitrance of lignocellulose), Ammonia-bases pre-treatments (SAA; soaking aqueous ammonia, ARP; ammonia recycle percolation, and AFEX; ammonia fibre explosion), CO_2 explosion and Oxidative pre-treatment (Kumar and Sharma, 2017).

Steam explosion/auto-hydrolysis is a conventional method used in pre-treating lignocellulosic substrates, which creates the partial hydrolysis of lignocellulose such that the cellulose sites are exposed for further enzymatic hydrolysis (Agbor *et al.*, 2011).It also causes hydrolysis of hemicellulose to sugars, e.g. xylose and glucose (Mosier *et al.*, 2005). Liquid hot water leads to lignin removal that exposes the sites of cellulose for further hydrolysis, as well as hemicellulose hydrolysis. Wet oxidation leads to hemicellulose degradation of smaller monomers and lignin oxidation, but it does not affect cellulose. SPORL is used to treat the substrate by reducing its size and through to the removal of lignin and hemicellulose fractions (Zhang *et al.*, 2013), therefore exposing cellulose for higher hydrolysis or conversion to glucose. AFEX causes cellulose swelling and reduces the crystallinity of cellulose, therefore leading to a higher release of carbohydrates or sugar yield after pre-treatment (Uppugundla *et al.*, 2014). ARP causes hemicellulose solubilisation but does not affect cellulose (Alvira *et al.*, 2010). CO_2 explosion disrupts the substrate structure, thus exposing more surface area for further hydrolysis (Kim and Hong, 2001; Zheng *et al.*, 2005). Oxidative pre-treatment acts by using oxidizing agents like oxygen, ozone, air and hydrogen peroxide (i.e. the most commonly used) in pre-treating lignocellulosic substrates (Nakamura *et al.*, 2004), which causes delignification by degrading lignin to acids (i.e. an inhibitory product). However, removal of the acids is required if such pre-treated substrates are to be used (Alvira *et al.*, 2010).

Some of the advantages of this method is that it requires low energy and reduced chemical cost due to low usage, is environmentally friendly and has no need for recycling expenses (steam explosion); it requires low temperature and low solvent cost, minimum production of inhibitory

products (liquid hot water); it is not expensive due to less CO_2 cost, requires low temperature, no toxic component is formed, and high robust capacity (CO_2 explosion); AFEX has no production of inhibitory compounds, and low processing cost as it requires no washing, no recovery and no reuse (Ammonia based pre-treatment) (Kumar and Sharma, 2017) .

The limitations are that the lignin-carbohydrates complexes are not completely degraded, inhibitory products might be produced at high temperature, there is the removal of hydrolysate by washing which causes loss in sugar yield-Steam explosion (Agbor *et al.*, 2011); it requires high energy caused by high amount of water needed- Hot liquid (Agbor *et al.*, 2011); oxygen might become combusted due to its nature and high cost of chemical - Wet oxidation (Bajpai, 2016); pre-treated substrates require a higher amount of water for washing, recovery cost of chemical used is high - SPORL (Bajpai, 2016); ARP requires high energy to maintain the temperature - Ammonia based pre-treatment; it requires a special reactor that can tolerate high pressure on a large scale and these reactors are costly - CO_2 explosion; it leads to more significant loss of hemicellulose - Oxidative pre-treatment (Lucas *et al.*, 2012).

1.2.7 *Chemical methods*

The use of chemical pre-treatment alone is capable of hydrolysing lignocellulose especially lignin, with or without enzymes, for the release of higher amounts of sugars compared to the physical methods (Sassner *et al.*, 2008; Kumar and Wyman, 2009; Xu *et al.*, 2009b; Digman *et al.*, 2010; Shuai *et al.*, 2010). It involves the use of dilute acids, sulphuric acid, dicarboxylic acids (oxalic and maleic acids), mild alkalis (hydroxyl derivatives of calcium, potassium, ammonium and sodium salts), ozonolysis, organosolv (methanol, acetone, ethanol, ethylene glycol), ionic liquids-ILs (imidazolium salts, cholinum amino acids, and cholium acetate), deep eutectic solvents (DESs) and natural deep eutectic solvents (NADESs) in reactors (Kumar and Sharma, 2017). Among the chemicals, inorganic acids (i.e. HCl and H_2SO_4) have been the most studied and productive chemicals. It also involves several post-pre-treatment processes such as washing for the removal of chemicals and recycling of chemicals to reduce chemical waste before the treated materials are used in animal feeding.

The use of dicarboxylic acids seem more favourable than acetic and sulphuric acids, in that these are less toxic to microorganisms and odourless. Also, these do not hinder glycolysis because they release less inhibitor, and so yield more sugars (Lee and Jeffries, 2011). Among the dicarboxylic acids, maleic acid gives additional benefits in that it leads to the hydrolysis of cellulose to glucose rather than degradation of glucose (Mosier *et al.*, 2002). The alkali degrades side chains of glycosides and esters, thus modifying lignin structure, de-crystallizing and

swelling cellulose, and dissolving hemicellulose (Cheng *et al.*, 2010; Sills and Gossett, 2011). Polysaccharide solubilisation by alkalis is lower than the acid pre-treatment that leads to the solubilisation of hemicellulose fraction (Sun and Cheng, 2002).

Ozonolysis does remove lignin but has a negligible effect on the polysaccharides, i.e. cellulose and hemicellulose (Kumar *et al.*, 2009). It is environmentally friendly because it does not produce toxic or inhibitory products, and it does not affect further processing like yeast fermentation and enzymatic hydrolysis that occurs after pre-treatment (Quesada *et al.*, 1999). Organosolv is used for lignin extraction, production and/or release of cellulose and hemicellulose. The lignin removal from the biomass exposes the fibres of cellulose for enzymatic hydrolysis (Agbor *et al.*, 2011). The ILs, DESs and NADESs act by disrupting the structural network of lignocellulose substrates by utilising the hydrogen bonds (Moulthrop *et al.*, 2005) found in the structure, thus reducing cellulose crystallinity and polymerization, as well as polysaccharides solubilisation.

The limitations involved with these methods are the generation of inhibitory compounds i.e. 5-hydroxymethylfurfural, aldehydes and phenolic acids (Saha *et al.*, 2005; Gírio *et al.*, 2010; Lee and Jeffries, 2011), corrosion of reactors or reaction vessels (Saha *et al.*, 2005; Lee and Jeffries, 2011), toxicity to microorganism if not adequately removed by washing after application (Agbor *et al.*, 2011). In fact, the removal process is very cumbersome, especially for alkalis which are costly (Sanchez and Cardona, 2008) and not environmental friendly (Keller *et al.*, 2003; Silverstein *et al.*, 2007).

1.2.8 *Biological methods*

These methods are most desirable and promising because these are considered as inexpensive and environmentally friendly, as they do not release any toxic or inhibitory substances. (Chen *et al.*, 2010; Sindhu *et al.*, 2016; Sharma *et al.*, 2017). The problem associated with these methods is that the process is slow and time-consuming (van Kuijk *et al.*, 2015; Sharma *et al.*, 2017). These methods involve the use of organisms such as bacteria, enzymes and fungi which are slow to grow when used for lignocellulosic pre-treatment as described below.

1.2.8.1 *Bacteria*

Lignocellulosic substrates are degraded by bacteria through the production of enzymes, e.g. cellulases, hemicellulases, and ligninases, with the majority of bacteria degrading hemicellulose and cellulose easily. However some lignin degrading bacteria have been discovered (Saritha and Arora, 2012; Lotfi, 2014; Sharma *et al.*, 2017). The selection of efficient bacteria is based on their ability to facilitate better enzymatic hydrolysis after pre-treatment. These bacteria

(*Butyrivibrio fibrisolvens, Pyrococcus furiosus, Clostridium stercorarium, Cellulomonas fimi, Acetivibrio cellulolyticus Ruminococcus flavefaciens, Ruminococcus albus,* and *Fusarium succinogenes etc.*) are anaerobic in nature and are known to secrete cellulases and hemicellulases (Miron *et al.*, 2001). Some of these anaerobic bacteria form extracellular complexes (i.e. multi-enzymes complexes), such as cellulolytic-hemicellulolytic ruminal enzymes complexes (Hyeon *et al.*, 2013). This type of cellulosome is more common with bacteria than yeast or fungi (Doi, 2008; Dashtban *et al.*, 2009), and the rate of degradation by this cellulosome is higher than the free enzymes (Van Dyk and Pletschke, 2012).

In addition, the bacteria belonging to the classes of γ- proteobacteria, actinomycetes, α – proteobacteria have been reported to degrade lignin by adopting the mechanism of cavitation (i.e. forming cavity), tunnelling and erosion mechanisms (Daniel *et al.*, 1987; Vicuna *et al.*, 1993; Blanchette, 1995; Daniel and Nilsson, 1998; Baldrian and Valášková, 2008; Li *et al.*, 2009; Eriksson *et al.*, 2012), as well as through enzymatic activity (Brown *et al.*, 2011; Bugg *et al.*, 2011). Bacteria belonging to the genus *Streptomyces* (Filamentous bacteria), *Pseudomonas sp,* and *Cellulomonas sp* have been found degrading and mineralizing lignin (Godden *et al.*, 1992; Berrocal *et al.*, 2000; Lynd *et al.*, 2002; Pérez *et al.*, 2002; Yang *et al.*, 2007) through enzymatic function. Many studies have shown that bacteria such as *Streptomyces viridosporus* T7A (Ramachandra *et al.*, 1988; Thomas and Crawford, 1998), *Nocardia* (Ramachandra *et al.*, 1988), *Rhodococcus jostii* RHAI (Ahmad *et al.*, 2011), *Pseudomonas paucimobilis* SYK-6 (Katayama *et al.*, 1988), *Pseudomonas putida mt-2* (Ahmad *et al.*, 2010), *Azotobacter sp* HM121 (Morii *et al.*, 1995), *Aneurinibacillus aneurinilyticus* (Raj *et al.*, 2007); *Bacillus sp* (Kunamneni *et al.*, 2007; El-Hanafy *et al.*, 2008), and *Paenibacillus sp* (Chandra *et al.*, 2007), can degrade, solubilise, depolymerise and mineralise the lignin molecule. Extracellular peroxidase is usually produced by *Actinomycetes* e.g. lignin peroxidase (Pasti *et al.*, 1991), for instance, the use of six strains of *Actinomycetes* in an experiment, led to the secretion of extracellular peroxidase and catalase activity. de Gonzalo *et al.* (2016) reviewed many lignin degrading bacterial enzymes including dye-decolorizing peroxidase (DyPs) a newly identified peroxidase (van Bloois *et al.*, 2010; Colpa *et al.*, 2014; Singh and Eltis, 2015; Yoshida and Sugano, 2015; Lambertz *et al.*, 2016); laccases (Santhanam *et al.*, 2011; Fernandes *et al.*, 2014; Chandra and Chowdhary, 2015; Martins *et al.*, 2015); glutathione-dependent β-etherases (Masai *et al.*, 1999; Sato *et al.*, 2009; Tanamura *et al.*, 2011); superoxide dismutases-MnSODs (Rashid *et al.*, 2015); catalase peroxidase – Amyco1 (Brown *et al.*, 2011) and dioxygenases (Bianchetti *et al.*, 2013).

The use of bacteria in lignocellulose pre-treatment has been reported to be successful but their enzymatic activity is low due to a less robust oxidative system (Hamelinck *et al.*, 2005; Li *et al.*, 2009; Brown and Chang, 2014) and incomplete lignin metabolism (Lotfi, 2014). Thus they exhibit longer time in achieving a lower amount of lignocellulose degradation when compared to fungi (Lotfi, 2014). Therefore, the use of bacteria is limited as a biological degrader of lignocellulosic materials.

1.2.8.2 *Enzymes*

Efficient and effective degradation of lignocellulosic substrates involves the use of two or more enzymes; this is due to the structural complexity of the substrate. Three major enzymes are usually available for obtaining soluble carbohydrates from the substrate and these include cellulases (cellulose degrading enzymes), hemicellulases (hemicellulose degrading enzymes) and ligninases (lignin degrading enzymes). These enzymes are described below where lignin degrading enzymes are discussed first.

1) Lignin degrading enzymes: These enzymes are involved in the depolymerisation of lignin, e.g. laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), sugar oxidase, quinone oxidoreductase, alcohol oxidase and glycol oxidase (Sarikaya and Ladisch, 1997).

Lignin peroxidase (LiP) is a glycoprotein that has a molecular mass of 38-48KDa and "heme" as a prosthetic group at its active centre. The high molecular size of its protein makes its degradation of lignin to take place in the visible lumen regions because it can't go further into the plant cell wall (Pérez *et al.*, 2002). In the presence of organic peroxide or hydrogen peroxide with LiP, it is oxidised in such a way that the substrate molecules lose two electrons to form compound I; an intermediate product (Lundell *et al.*, 2010). Also, the lignin aromatic nuclei lose one electron to the oxidised LiP to form cation radicals. A reaction occurs between these radicals and oxygen and nucleophiles leading to the breakdown of C-C and C-O bonds, which facilitates the depolymerisation of lignin and exposure of the aromatic ring. The compound formed from the depolymerisation of lignin is oxidised on entry into the hyphae. The intermediate "Compound I" form of LiP produces a radical cation "veratryl alcohol" which initiates the cleaving of various lignin linkages. Finally, better lignin decomposition is observed by the products of reaction (i.e. active oxygen species) which oxidise the lignin molecule. The reaction is usually between the radicals and oxygen (Kirk and Farrell, 1987; Sarikaya and Ladisch, 1997).

Manganese dependent peroxidase (MnP) is a glycosylated protein having a higher molecular mass of 45-60KDa which is slightly similar to LiPs and it also contains a prosthetic group called

"heme" (Pérez *et al.*, 2002). It has some catalytic cycle that is similar to that of LiPs, but differences in the catalytic cycle occur with the other form of peroxidases. MnP utilises Mn^{2+} for its catalytic reaction, which has high availability in lignocellulosic substrates. Initiation of the MnP catalytic cycle begins by native ferric enzyme binding to hydrogen peroxide to form a complex "iron-peroxide". MnP compound I is produced by the heme of the MnP donating two electrons. The removal of the di-oxygen bonds leads to a water molecule explosion, and further reduction of MnP compound I to compound II (Mn^{2+}) is obtained. The compound II (i.e. monochealated Mn^{2+}) is oxidised to Mn^{3+} by donating an electron. Similarly, another Mn^{3+} is formed through the reduction of compound II. This further leads to water molecule explosion and native enzyme formation (Hofrichter, 2002). The Mn^{3+} formed acts in the lignocellulose as a very active oxidant by oxidising phenolic compounds. In most cases, it does not oxidise non-phenolic units. The various reactions undergone by phenoxy-radicals produced by MnP lead to lignin polymerisation (Pérez *et al.*, 2002). The presence of hydrogen peroxide in high concentrations can lead to the production of compound III. This compound usually inactivates the activities of the enzyme therefore, limiting its efficiency (Wariishi *et al.*, 1988).

Laccase is a glycoprotein that has a molecular size ranging from 60 -70DKa and this enzyme exists both inside and outside the plant cell wall if obtained from Basidiomycetes (Baldrian and Šnajdr, 2006). It is an oxidase of multiple coppers containing polyphenol, accelerating the oxidative reaction through the transfer of electrons of lower- redox potential compounds representing primarily phenolic compounds. For instance, the presence of mediator 2, 2-azino-bis ABTS (3-ethylbenzothiazoline-6-sulphonic acid) enables laccase to oxidise non-phenolic units. Oxidation of the nucleus of phenolic compounds occurs by an electron removal, leading to the formation of a phenoxy free radical. The radicals under several reactions (i.e. non enzymatic reactions) break the lignin polymer and further lead to the breakdown of alkyl-phenyl, oxidation of Cα and demethoxylation (Pérez *et al.*, 2002). Consequently this breakdown is very significant in the degradation of lignin phenolic groups and the release of phenolic during lignin fermentation (Sarikaya and Ladisch, 1997).

Glycol oxidase, sugar oxidase and alcohol oxidase are also lignin degraders and are usually diverse in nature, i.e. they assist other lignin-degrading enzymes for their proper function. For instance, H_2O_2 production requires these enzymes while H_2O_2 is very important in the activity of ligninase. H_2O_2 oxidises the ligninase enzymes to facilitate their efficiency (Sarikaya and Ladisch, 1997).

2) Cellulose degrading enzymes: Cellulose occupies about half of the content of lignocellulose biomass (Ryu and Mandels, 1980). This indicates that efficient degradation of cellulose is vital

when the whole degradation of lignocellulosic substrate is considered. Three major groups of cellulases involved in the hydrolysis of cellulose include cellobiohydrolases (exogluconases), carboxymethylcellulases (endoglucanases) and cellobiases (β-glucosidases) (Singhania *et al.*, 2010).

Carboxymethylcellulases are enzymes that randomly break down the cellulose chains, especially the swollen amorphotic regions of the chain. This hydrolysis decreases the length of the chain and also slowly increases the reducing groups' number. The end product (fibrolytic short chain cellulose) obtained from degradation by carboxymethylcellulases on cellobiose and crystalline cellulose, is a reflection that the enzyme is not capable of degrading the compounds (Ryu and Mandels, 1980). The addition of cellobiohydrolases is required at this junction.

Cellobiohydrolases (CBH) are exogenous and exist in two forms: CBH I and CBH II. These act by breaking the cellulose molecule from both ends. CBH I acts further by increasing the number of reducing groups through the removal of cellobiose and glucose molecules from the cellulose unit ends. CBH II acts by breaking the carbohydrate from the reduced ends. Cellulose crystalline fractions are efficiently degraded by the CBHs alone, since carboxymethylcellulase degrades the swollen amorphotic regions (Pérez *et al.*, 2002). Apart from this, they occupy about 40 -70% of the totality of all cellulose activities.

The β-glucosidase enzyme acts by breaking the linkages of β-1, 4 glycoside down to glucose units. To obtain complete cellulose hydrolysis, the cellobiose produced from cellobiohydrolases needs to be reduced by the exogenous β-glucosidases to glucose units. When β-glucosidase is not present in the cellulose hydrolysis process it is added (Sanchez and Cardona, 2008). Further breakdown of cellobiose by cellobiohydrolases is usually limited due to inhibition posed by the high concentration of cellobiose on the enzyme CBHs. In an enzyme preparation, a different number of cellulases may exist, and this depends on its fungal origin. For instance, six different carboxymethylcellulases were found in *Trichoderma reesei* (Sanchez and Cardona, 2008).

Classification of cellulases into families is dependent on the sequence of their amino-acids and catalytic domain similarities. The catalytic domain is where the active site "heme" is found, and this is where substrate breakdown begins. The cellulase binding to the insoluble fraction of the substrates is facilitated by the cellulose binding domain (CBD) (Linder and Teeri, 1997). Three domains exist in the cellulases (Hall *et al.*, 2011): Catalytic domain, Cellulose binding domain, and Glycosylated-flexible domain, which links the catalytic and the cellulose binding domains together. Through the sequencing of amino-acids, about 12 and 14 families of catalytic domains

and CBDs respectively have been identified (Duff and Murray, 1996; Levy and Shoseyov, 2002).

3) Hemicellulose degrading enzymes: There are several enzymes involved in the degradation of hemicellulose such as: β-D-glucosidase, exoxylanase, endoxylanase, endoarabinanase, α-D-galactosidase, endomannanase, endogalactanase, α-L-arabinosidase, β-D-mannosidase, α-1,2-L-fucosidase, β-D-galactosidase, β-D-xylosidase, acetylgalactan esterase, acetylxylan esterase, acetylmannan esterase, α-D-glucuronidase, ferulic acid esterase and courmaric acid esterase (Coughlan *et al.*, 1993). Hemicellulose is heterogeneous when compared to cellulose; it requires more enzymatic reactions for better degradation and fermentation of lignocellulosic substrates (Malherbe and Cloete, 2002).

The enzymatic activity involved in the degradation of hemicellulose is grouped into two, based on its catalytic reaction (Shallom and Shoham, 2003), and this includes: Glycoside hydrolase (breaks down the glycosidic bonds) and Carbohydrate esterase (breaks down the acetate side groups or ferrulic acid ester linkages). Xylan is a major hemicellulose carbohydrate usually degraded by xylanases and accessory enzymes that are related. The breakdown of β-1, 4-bonds into branched and randomly sized xylo-oligomers is obtained through the action of endo 1, 4-β-xylanase. Also, α-L- arabinose and arabinofuranosidase (accessory enzymes) remove L-arabinose from arabinofuranosyl by degrading it to attain the contained hemicellulose.

On the xylose moieties, there exist O- acetyl groups and, also, there are ester bonds that are found between ferulic acid and arabinose substitution. The O –acetyl groups and ester bonds are broken down by xylan esterase, p-coumaric and ferulic esterase (i.e. hemicellulolytic esterase) (Shallom and Shoham, 2003; Gírio *et al.*, 2010).

Furthermore, β-1, 4 -xylosidase hydrolyses xylooligomers (i.e a product from xylanase) into single units of xylose (Sánchez, 2009) while β-1, 4 manno-oligomer (i.e. a product obtained from β- mannan) was hydrolysed by β-mannosidase to produce single units of mannan. To obtain an efficient breakdown of a particular hemicellulolytic chain, some hemicellulolytic enzymes are needed to co-function. For instance, complete degradation of O –acetyl-4-0-methyl glucuronoxylan, requires about four different enzymes (β-xylosidase, acetyl esterase, endo-1, 4-β-xylanase and α-D-glucuronidase) in co-existence (Pérez *et al.*, 2002).

1.2.9 *Aerobic Fungi*

Filamentous fungi (Basidiomycetes) are the major specialised group involved in the degradation of lignocellulosic substrates (Keller *et al.*, 2003; Sindhu *et al.*, 2016). By their fungal lifestyle and the habitat, they can be classified into three namely: litter decomposing

fungi, mycorrhizal fungi and wood decaying fungi. The most commonly used are the wood decaying fungi and these fungi are further sub divided into three based on the fraction of plant cell wall they attack and on the product obtained from the attack; they include: Brown rot, Soft rot and White rot fungi. Also, some other filamentous fungi such as *Aspergillus* spp. and *Penicillium* spp. are identified due to their efficient ability in the breakdown of hemicellulose and cellulose in the plant cell walls that are obtained from agricultural residues and forestry products.

1.2.9.1 *Brown rot fungi (BRF)*

Taxonomically, brown rot fungi are similar to white rot fungi (Hatakka, 2005) and the two belong to the same division. Brown rot fungi degrade hemicellulose and cellulose with little effect on lignin (Eriksson *et al.*, 2012). The main effect these fungi have on wood is that the wood turns brown while still maintaining its shape, during which the cellulose and hemicellulose are degraded, e.g. *Gloeophyllum trabeum*, *Lenzites trabea*, *Coniphora putenana*, *Serpula lacrymans*, *Meruliporia incrassate*, *Fomitopsis lilacano*, *Agrocybe aegerita* and *Flammulina velutipes* (Blanchette, 1995). These fungal treatments had no positive effect on digestibility of the substrate but rather reduced the digestibility in some experiments when compared with an untreated straw (Zadrazil *et al.*, 1999b). The possible reduction may be because the fungi are using the available nutrients for their growth. This may mean that these fungi were non-selective in their degradative action. Also, they act by the hyphae penetrating wood cells, such that this penetration leads to the diffusion of protein with small molecular weight; penetration after that leads to better hydrolysis of the lignocellulosic substrates (Shimada *et al.*, 1997).

Lignin depolymerisation by these fungi is achieved through further penetration of the hyphae through the existing plant spore spaces into the various plant cell walls. The fungal hyphae are usually found in the cell lumen where the diffusion begins and the hyphae are found closely connected to the secondary cell wall (S3) inner layer. Decaying of the substrates starts from the secondary cell wall (S2) middle layer (Eriksson *et al.*, 2012). There are 7 layers in the plant cell (Butterfield, 2012) and these include the following: Middle lamella, primary wall, secondary wall (S1) outer layer, secondary wall (S2) middle layer, secondary wall (S3) inner layer, helical thickening layer, and warty layer.

1.2.9.2 *Soft rot fungi (SRF)*

Extensive degradation by soft rot fungi causes the mechanical stability of the wood to be lost as well as making it wet. Soft-rot fungi degrade polysaccharides (i.e. hemicellulose and cellulose) without much effect on lignin, leaving the lignocellulosic material watery soft

(Chahal, 1981). These fungi belong to the phylum Deuteromycota and Ascomycota. Typical examples of soft rot fungi are *Kretzchmaria deusta*, *Cadophora* spp., *Chrysonilia sitophila*, *Chaetomium funicola* and *Daldinia concentric.* Degradation by soft rot fungi exists in 2 major forms, with the different forms occurring in the secondary cell walls (Blanchette, 2000): type 1 degradation leads to the formation of long horizontal cavities in the plant secondary cell wall, and type 2 degradation causes erosion in the secondary cell wall

1.2.9.3 *White rot fungi (WRF)*

These fungi act on the secondary cell wall of plants that are low in nutrients, through their various enzymes such as laccases, hemicellulases, cellulases, manganese peroxidase and lignin peroxidase (Hatakka, 2005). Also, some of these fungi degrade polysaccharides without enzymatic activities (Baldrian and Valášková, 2008). The ligninolytic enzymatic activity exhibited by these fungi was used for its classification (Hatakka, 1994). The three (3) main classifications are those producing enzyme laccase, MnP and LiP; those producing enzyme laccase and LiP; and those producing enzyme laccase and MnP.

The degree to which these fungi degrade lignocellulosic substrates varies considerably, even under the same growth conditions (Eriksson *et al.*, 2012). The major attribute of most of these fungi is the colonisation of plant cell lumina; where different zones of the plant cell are more closely degraded, these zones subsequently unite together to form higher degraded zones. The voids created by these zones undergo a non-selective degradation process where the voids are filled with the fungal mycelia. These fungi (*Abortiporus biennis, Dichomitus squalens* (Rytioja *et al.*, 2017)*, Pleurotus sp., Agaricus bisporus, Crinipellis sp., Coriolus versicolor, Poria placenta, Lenzites striata* etc.), have the tendencies of degrading lignin with little effect on polysaccharides (Zadražil and Brunnert, 1982a; Tuyen *et al.*, 2012). These fungi bring about a white-mottled substrate rot and usually break the C-4 ether bonds by cleaving the aromatic ring of Cα – Cβ, therefore, increasing the *in vitro* digestibility of the substrate (Zadrazil *et al.*, 1999b).

Some WRF act non-selectively by degrading the polysaccharides while others act selectively by degrading lignin with less effect on the polysaccharides (Blanchette, 1995). However, some of these fungi, e.g. *Ganoderma applanatum*, are capable of carrying out both of the effects (i.e. degrade lignin, cellulose and hemicellulose together). The selective white rot fungi includes *Pleurotus* sp (Castoldi *et al.*, 2014), *Ceriporiopsis subvermispora* (Wan and Li, 2011; Cianchetta *et al.*, 2014; Hori *et al.*, 2014); *Punctularia sp* (Suhara *et al.*, 2012); *Irpex lacteus* (Xu *et al.*, 2010; García-Torreiro *et al.*, 2016; Yao *et al.*, 2017; Qin *et al.*, 2018). Fungi like

this are usually discouraged from use because they degrade the useful components needed for biotechnology applications and animal feeding. The effects of *Phlebia radiate (Kuuskeri et al., 2016), Pleurotus eryngii* and *Ceriporiopsis subvermispora* on lignin straw varies. *Phlebia radiata* acts on the secondary plant cell wall while *P.eryngii* and *C. subvermispora* partially act on the middle lamella (Burlat *et al.*, 1998). Fungi such as *Pleurotus ostreatus*, *Phanerochaeta chrysosporium, Pleurotus eryngii* and *Ceriporiopsis subvermispora* have been identified as suitable fungi for biopulping purposes (Hatakka, 2005).

Most hardwood and grass angiosperms and gymnosperms contain syringyl units and guaicyl units whereas softwoods contain only guaicyl. WRF act better on syringyl units because it is more easily degraded than the guaicyl lignin type, therefore making these fungi act better on hardwoods than softwoods (Hatakka, 2005; Singh and Chen, 2008).

1.2.9.4 *Other lignocellulolytic fungi*

Other fungi of noticeable effect due to the production of a high volume of heterogeneous enzymes, e.g. *Trichoderma* spp., and *Aspergillus* spp., exist in several ecosystems because their metabolism is very flexible and, also, they degrade a large number of nitrogen and carbon sources (Flipphi *et al.*, 2009). The most studied and industrially utilised family under this genus "*Aspergilli*" are *A. oryzae* and *A. niger* (Hu *et al.*, 2011). These two families are capable of degrading hemicellulose and cellulose with their enzymatic activities such as xylanase, galactomannanase, cellobiohydrolase, pectinase, β-glucosidase and endoglucanase (de Vries and Visser, 2001). Some of the *Aspergilli* have been found degrading some aromatic compounds linked to lignin (Duarte and Costa-Ferreira, 1994).

In the production of commercially-available hemicellulolytic and cellulolytic enzymes *Trichoderma reesei* has been used extensively worldwide (Sánchez, 2009). The enzymes produced by *T. reesei* include endoglucanase, cellobiohydrolase, and in few cases cellobiase (Kim *et al.*, 1997). The enzyme cellobiohydrolase is usually inhibited by accumulated cellobiose produced by cellobiase (Fang *et al.*, 2010).

In conclusion, the use of fungi has been the current trend for improving lignocellulosic substrates, therefore improving intake and digestibility (Zhang *et al.*, 2007; Yu *et al.*, 2009a) and upgrading them for animal feeding (Villas-Bôas *et al.*, 2002). The major problem with this method is the breakdown of cellulose and hemicellulose (i.e. useful polysaccharides) that are usually bound to lignin during the process of delignification by some fungi. For this reason, the most effective organism should be an organism that degrades lignin with little or minimal effect on hemicellulose and cellulose. Also, when the focus of substrate degradation is for animal

feeding, lignin should be targeted and not polysaccharides for the possible release of nutrients from the lignocellulosic complexes (Zadrazil *et al.*, 1999b). This tends to increase the number of nutrients available for the growth of rumen microbes from which microbial protein can be obtained (Villas-Bôas *et al.*, 2002).

Fungi can be used for this purpose because they exhibit enzymatic function which is usually presented in the form of laccase, lignin peroxidase, manganese peroxidase (Arora *et al.*, 2002; Arora and Sharma, 2010), hemicellulases and cellulases etc. However, those fungi that produce cellulase and hemicellulase do lead to higher loss of organic matter, since they degrade the essential nutrients needed by the animals (Kerem *et al.*, 1992; Deshpande *et al.*, 2009).Various research reports, where brown-rot and soft–rot fungi were used for upgrading substrates for ruminant production, have observed reduced digestibility because the fungi were reported to have utilised the available nutrients, i.e. cellulose and hemicellulose, for their growth (Jung *et al.*, 1992; Karunanandaa *et al.*, 1995). However, some strains of white rot fungi have been shown to improve the digestibility of most straws (Jalč *et al.*, 1994; Díaz and C. Sánchez, 2002) while selectively degrading lignin (Akhtar *et al.*, 1997; Yu *et al.*, 2009a).

1.2.10 *Combined pre-treatment*

Several researchers have identified that combination of more than one pre-treatment leads to a better release of sugars, reduction in pre-treatment time, higher lignin degradation, improved enzymatic hydrolysis, and it seems more promising in the pre-treatment of lignocellulose substrates than the use of only one pre-treatment (Sindhu *et al.*, 2016). The combination of a physical method with chemical methods or enzymes led to higher sugar yields as the chemical and enzymes solubilise lignin. The combination of physical methods with chemical methods have been investigated by several researchers (Fan *et al.*, 1987; Sun and Tomkinson, 2002; Keshwani and Cheng, 2010; Ma *et al.*, 2010; Chen *et al.*, 2011a; Chen *et al.*, 2011b; Lu *et al.*, 2011; Xu *et al.*, 2011; Zakaria *et al.*, 2014; Zhu *et al.*, 2015; Zhu *et al.*, 2016) and with enzymes (Karunanithy and Muthukumarappan, 2010; Karunanithy *et al.*, 2012). Also, the combination of two or more chemicals yielded higher sugar than with the use of only one chemical pre-treatment (Teixeira *et al.*, 1999; Kim *et al.*, 2011a; Kim *et al.*, 2011b; Mesa *et al.*, 2011; Geng *et al.*, 2012).

Several researchers have also investigated the combined effect of the use of physical or chemical with biological treatment using white rot fungi (Kadimaliev *et al.*, 2003; Balan *et al.*, 2008; Yu *et al.*, 2009b; Ma *et al.*, 2010) and/or physicochemical with biological pre-treatment using white rot fungi (Sawada *et al.*, 1995; Wang *et al.*, 2012) on lignocellulosic substrates.

The combined effects led to the maximization of the release of hemicellulose, higher sugar yields and increase in lignin removal.

Also, several researchers have combined the use of 2 biological agents (co-culture) in pre-treating lignocellulose substrates which has led to higher lignin degradation than only one biological agent (monoculture). Asiegbu *et al.* (1995) used three fungi (i.e. *P. chrysosporium*, *T. versicolor*, and *P. ostreatus*) in pre-treating spruce sawdust, and Chi *et al.* (2007) used *P. ostreatus* with *Physisporinus rivolus* or *C. subvermispora* in pre-treating aspen wood.

1.2.11 *Feed additives to improve utilization of lignocellulosic substrates for increased animal performance*

In general, feed additives are used to improve voluntary feed intake by increasing palatability as well as feed utilization, feed digestibility, animal feed safety, animal performance regarding growth or production, and animal health (e.g. prevention of diarrhoea in calves and lamb at the neonatal stage of life). These are achieved by the feed additives manipulating the rumen microbial ecosystem and enhancing fibre degradation with the aim to facilitate desirable and efficient fermentation, reducing ruminal disorders, modifying the small intestine flora, facilitating the developmental rate of rumen flora and fauna, and removing pathogens (Wallace and Newbold, 1995; Bodas *et al.*, 2008b; Elghandour *et al.*, 2015).

Feed additives can be grouped into chemical (i.e. methane inhibitors, ionophores, monensin, antibiotics, and defaunating agents), natural products (i.e. probiotics, prebiotics, dicarboxylic acids, plants), plant extracts such as tannin, saponins and essential oils, and microbial (i.e. filtrates and cell fragments, yeast and fungi) feed additives (Knowlton *et al.*, 2002; Jouany and Morgavi, 2007; Elghandour *et al.*, 2015; Nagpal *et al.*, 2015; Puniya *et al.*, 2015; Zeng *et al.*, 2015; Belanche *et al.*, 2016). These feed additives in ruminants are regarded as effective if they are capable of performing multiple functions. For example, reduction of methane production by favouring higher propionate at the expense of acetate in the fermentation pathway, facilitation of higher protein bypass from the rumen to increase its bioavailability in the true stomach, reduction in the degradation of highly fermentable carbohydrates in the rumen that might reduce the pH thus regulate lactic acid concentration in the rumen and improvement of fibre degradation and/or digestion are considered helpful in increasing intake, growth and production of animals (Jouany and Morgavi, 2007; Seo *et al.*, 2010; Elghandour *et al.*, 2015; Puniya *et al.*, 2015; Azzaz *et al.*, 2016).

The chemical additives were capable of carrying out almost all of these functions, but their use was limited as these were not safe in the supply food chain for consumers due to their potential

residues in animal products. These residues may be detrimental to humans as well as the environment due to their toxicity to the host animals, and antibiotic resistance in human bacteria due to their prolonged usage in animal diets (Barton, 2000; Yang and Carlson, 2004; Manero *et al.*, 2006; Parveen *et al.*, 2006; Salem *et al.*, 2014).

The natural products were then used as suitable alternatives for chemicals, especially antibiotics, (Fon and Nsahlai, 2013; Durmic *et al.*, 2014) as they are safe in the food supply chain for consumers but they are limited in that no precise dose is available and, if overdosed, they can be toxic to rumen microbes which can negatively affect the performance of the animals. Their efficacy cannot be ascertained as for antibiotics, as it can be influenced by the diets and/or animal physiological conditions (Jouany and Morgavi, 2007; Bodas *et al.*, 2008a).

The microbial feed additives are also called probiotics or direct fed microbials (DFM) and they seem to be more promising, in that they are capable of carrying out almost all the functions of both natural products as well as chemical additives (Elghandour *et al.*, 2015; Nagpal *et al.*, 2015; Azzaz *et al.*, 2016). In addition, they can facilitate the growth of other microbes as well as release enzymes, especially cellulose, involved in fibre degradation in the rumen, thus enabling better utilisation of fibrous feed. They are usually commercially applied as a gel, paste, capsule or powder (Puniya *et al.*, 2015), as well as locally applied as live cultures given orally or intra-ruminally (Dey *et al.*, 2004). The limitations of this method are that there is a low viability of these microbes when exposed to a longer period of storage and too harsh field conditions. Most of these microbes can't withstand harsh processing techniques, such as the use of heat when pelletizing (Azzaz *et al.*, 2016). Among the microbes, yeasts and fungi are mostly used in adult ruminants rather than bacteria (Wallace and Newbold, 1995), which is because most of the bacteria (*Lactobaccillus streptococcus*, *Lactobacillus acidophilus*, *Propionibacterium* spp., *Bacillus* sp., *Bifidobacterium* spp., *Streptococcus* spp., *Prevotellabryantii* sp., *Enterococcus*, are *Megasphaera elsdenii)* are either lactic acid producing or utilizing bacteria used in preventing acidosis caused by high amount of lactic acid in the rumen (Elghandour *et al.*, 2015; Nagpal *et al.*, 2015; Azzaz *et al.*, 2016), which is among the functions of yeast and fungi in the rumen. The yeast and fungi are capable of enhancing the growth and activities of total bacteria and specific groups of bacteria (i.e. cellulolytic and lactic acid utilizing) involved in better utilisation of feed in the rumen (Wallace and Newbold, 1995; Callaway and Martin, 1997; Beharka and Nagaraja, 1998; Elghandour *et al.*, 2015; Azzaz *et al.*, 2016) and hence can increase the amount of microbial protein that flows from the rumen to the small intestine (Wallace and Newbold, 1995). Fungi secrete enzymes that have higher oxidative function than bacteria (Azzaz *et al.*, 2016). Further discussions of yeast are given below.

1.2.11.1 *Yeast*

These are usually present as live cells (in freeze-dried condition) or as cultures (i.e. still growing in a media) and in these forms they are added to feed to increase its palatability, therefore increasing intake and degradability in the rumen (Ando *et al.*, 2005) as well as enhancing nutrient digestibility (El-Ghani, 2004). Different yeasts (e.g. *Scheffersomyces stipites, Klyveromyces marxianus* and *Saccharomyces cerevisiae*) exist, but *S. cerevisiae* has been the most widely studied and most active, especially in the production of ethanol (Wallace and Newbold, 1993; Chandel *et al.*, 2015; Puniya *et al.*, 2015). *S. cerevisiae* is capable of utilising sugars (e.g. glucose, fructose, mannose, sucrose, galactose and maltose) with the exception of arabinose and xylose (van Maris *et al.*, 2006). It can grow both in the anaerobic and aerobic environment, but when raised in an anaerobic environment, unsaturated fatty acids and ergosterol were not produced; therefore supplementation of these compounds is required for better growth under anaerobic conditions (Panda, 2011).

Availability of *S. cerevisiae* in feed stimulates the development and activities of lactate utilizing bacteria in the rumen (Arambel and Kent, 1990; Dawson, 1993; Martin and Streeter, 1995; Newbold *et al.*, 1996; Beauchemin *et al.*, 2003; Elghandour *et al.*, 2015; Nagpal *et al.*, 2015) and aids in the removal of oxygen in the rumen that may be toxic to the bacteria, thus making the rumen more anaerobic (Arambel and Rung-Syin, 1987; Wallace and Newbold, 1995; Newbold *et al.*, 1996; Kutasi *et al.*, 2004; Azzaz *et al.*, 2016). Consequently, it aids in maintaining the pH of the rumen, even when animals are fed with highly fermenting substrates, because of its buffering capacity preventing lactic acid accumulation (Kung Jr, 1996; Chaucheyras-Durand *et al.*, 2008; Elghandour *et al.*, 2015) and thus improving the utilization of high grain diets as well as low quality forages when fed in the diet of ruminants (Arambel and Kent, 1990).

Furthermore, the inclusion of yeast in ruminant feed improves fibre digestion by stimulating the activities of rumen cellulolytic bacteria as well as increasing the amount of microbial protein that gets into the intestine (Newbold *et al.*, 1996; Jouany and Morgavi, 2007). The drawback with the use of yeast culture is that, before stimulation of ruminal activity especially bacteria by yeast can be obtained, it requires a growth medium that is rich in vitamin B, amino acids and organic acids which are very expensive to obtain (Callaway and Martin, 1997) In addition to this, *S. cerevisiae*, the most studied yeast, has been found not to be capable of degrading sugars such as arabinose and xylose (Demeke *et al.*, 2013; Diao *et al.*, 2013); growth is limited in the rumen environment due to the higher temperature and chemical composition of the rumen (Elghandour *et al.*, 2015); inconsistency in their response towards the release of volatile fatty

acids is recorded (Elghandour *et al.*, 2015); they do not increase the growth of anaerobic fungi in most cases, therefore limiting their use in ruminant production.

1.2.11.2 *Fungi (Aerobic and Anaerobic Rumen fungi)*

The fungi used as additives can either be aerobic or anaerobic rumen fungi and they have been extensively used in improving the utilization of feed in ruminant animals (Elghandour *et al.*, 2015; Puniya *et al.*, 2015). They are more efficient than yeast, in that they are capable of utilising more sugars due to their higher enzymatic function than yeast and bacteria (Elghandour *et al.*, 2015).

The most commonly used aerobic fungi are *Aspergillus oryzae* (i.e. aerobic fungi) and these are mostly applied by sprinkling on feed, mixing it with other feed components to make a compound diet, or used as an inoculant in silage production (Wallace and Newbold, 1995). The use of the fungus in animal diets increased the feed intake and nutrient degradability (Caton *et al.*, 1993; Tricarico *et al.*, 2007); increased milk yield and production (Williams and Newbold, 1990; Higginbotham *et al.*, 1993), increased weight gain (Sun *et al.*, 2017); improved lactate utilization by *Selenomonas ruminantium* (i.e. a rumen microbe) by providing the bacteria with malic acid (i.e. a dicarboxylic acid), a component needed for its growth (Martin and Streeter, 1995); increased the population of total ruminal anaerobic bacteria and mostly the cellulolytic bacteria (Wallace and Newbold, 1995; Kung Jr, 1996; Seo *et al.*, 2010); increased the release of volatile fatty acids and ammonia (Gordon *et al.*, 2016; Sun *et al.*, 2017); prevented excess ruminal lactic acid accumulation (Wallace and Newbold, 1995; Puniya *et al.*, 2015); and facilitated weaning in calves (Beharka *et al.*, 1991; Puniya *et al.*, 2015). However, its use is limited in that there has been inconsistency in the positive responses obtained from the use of this fungus, as its use led to no increase in milk yield and production, no increased intake and digestibility by some researchers (Yohe *et al.*, 2015; Zicarelli *et al.*, 2016); also in most cases it doesn't support the increase in rumen fungi involved in fibre digestion (Wallace and Newbold, 1995).

More recently, the use of the ARF has received more attention in that, apart from the above benefits mentioned with the use of the aerobic fungi, ARF use in ruminant diets has been capable of producing a more consistent response in improving ruminant productivity during feeding trials (Puniya *et al.*, 2015); increasing the total rumen fungal population and activities (Chang *et al.*, 1999) thus leading to an efficient fibre digestion; and increasing the sites needed for bacteria degradation through their colonization and enzymatic function (Dagar *et al.*, 2011) thus supporting further fibre hydrolysis. Also, they proliferate quickly as they are healthy inhabitants of the rumen which make ARF more promising than the aerobic fungi and yeast

(Puniya *et al.*, 2015).These fungi are capable of increasing the animal performance regarding milk and meat production, increased feed intake and digestibility etc. (Lee *et al.*, 2000a; Dey *et al.*, 2004; Thareja *et al.*, 2006). They are usually applied orally (Dey *et al.*, 2004), intra-ruminally (Lee *et al.*, 2000a) as well as inoculants in silage production (Lee *et al.*, 2015).

ARF derive their energy from a variety of soluble sugars and the way in which they utilise the sugar is dependent upon their species and strain. Fungi utilise different polysaccharides (i.e. cellulose, xylan, pullulan, pustulan, inulin and starch) and polygalacturonate. Various fungal strains that exist are *Neocallimastix, Piromyces, Caecomyces, Orpinomyces, Anaeromyces* and *Cyllamyces* strains (Sehgal and Singh; Barr *et al.*, 1989; Gordon and Phillips, 1989; Bernalier *et al.*, 1990; Breton *et al.*, 1990; Orpin and Joblin, 1997).

Rumen fungi further break down cellulose, hemicellulose and hydrolyse free oligosaccharides during their vegetative stage of growth through their enzymatic activities (Williams and Orpin, 1987; Wilson and Hatfield, 1997). They produce a wide range of enzymes e.g. esterases i.e. p-coumaroyl esterase, feruloyl esterase and acetyl esterase (Blum *et al.*, 1999; Yue *et al.*, 2009a); cellulases i.e. endoglucanases, exoglucanase, β –glucosidase, β-fructosidase, β-L-arabinofuranosidase, and β-xylosidase (Atanasova-Pancevska and Kungulovski, 2008; Comlekcioglu *et al.*, 2010); hemicellulases i.e. xylanases and mannanase (Coughlan and Hazlewood, 1993; Novotna *et al.*, 2010); proteases (Wallace and Joblin, 1985a); and chitinases (Sakurada *et al.*, 1995; Novotná *et al.*, 2008) which break down different polysaccharides (Gordon and Phillips, 1998; Ho and Abdullah, 1999; Denman and McSweeney, 2006; Leis *et al.*, 2014; Puniya *et al.*, 2015). However, the presence of glucose and xylose in the rumen limits the enzymatic activity of some fungi, especially those producing cellulase and xylanase (Lowe *et al.*, 1987b; Hébraud and Fèvre, 1988; Ho and Abdullah, 1999). Extracellular metalloprotease production has been an additional extracellular activity of fungi (Wallace and Joblin, 1985b). Most strains of fungi produce formate, ethanol, acetate, and lactate, hydrogen (H_2) and carbon dioxide (CO_2) as their end products of degradation. However, *N. patriciacum* does not generate ethanol and formate as part of its degradation end products (Orpin and Munn, 1986). Since they produce acetate as part of their end products, there is the probability that the use of ARF as feed additives might lead to higher methane production.

1.2.12 *Mode of action/colonization of anaerobic rumen fungi (ARF)*

With the availability of thick or lignified plant materials in the rumen, the fungal motile zoospores preferentially colonise the different damaged parts of the ingested substrate within few minutes (i.e. 5-20) and get attached to the plant. However, the impaired parts vary

depending on the forage; in legumes (the epidermal damaged regions); in grasses (the hollow stem internal surface); and in leaves (majorly the surrounding areas of vascular bundles and sometimes they invade the stomata) (Bauchop, 1979b). After attachment to the plant, higher populations of the fungi are found attached within 2-3h, and the flagella get detached within a few minutes (Bauchop, 1979b), the zoospores encyst the substrate and begin to germinate and develop hyphae and/or rhizoids, i.e. a vegetative structure. The rhizoids support the development of sporangium, i.e. a reproductive structure (Lowe *et al.*, 1987a; Edwards *et al.*, 2008), which commences at 12h and by the end of the day the sporangium has grown to adult size and the reproductive cycle is completed. The fungal life cycle immediately begins again, with the sporangia releasing new motile zoospores. Fungal sporocyst growth has also been identified to vary, even in the presence of lignified substrate, and this can be attributed to selective colonization by different fungal species or substrate composition (Grenet and Barry, 1988; Grenet *et al.*, 1989).

1.2.13 *Aerobic fungal delignification of lignocellulose*

Several fungi (*Ganoderma applantum, Armillariella* spp) have been discovered in degrading wood components in the forest into a substance that could be used as animal feed (Tangol, 1976). These fungi increased the digestibility of the wood from 3% - 77% in some cases, but this was obtained at a very slow rate with high organic matter loss (Zadražil and Brunnert, 1982a).

Fungi such as *Phaenerochaeta chrysosporium* have the tendency of degrading lignin but un-selectively degrade cellulose and hemicellulose as well as lignin (Sharma *et al.*, 2010; Dong *et al.*, 2013). This leads to a higher organic matter loss which made this fungus un-acceptable for improving feed for animals (Jung *et al.*, 1992; Chang *et al.*, 2012; Syed and Yadav, 2012).

In order to limit the total loss of dry matter by non – selective white rot fungi, researchers have been able to identify some selective lignin degraders such as *Phlebia* species (Fackler *et al.*, 2006; Arora and Sharma, 2009a), *Ceriporiopsis subvermispora* (Guerra *et al.*, 2004; Fernandez-Fueyo *et al.*, 2012), *Physisporinus rivulosus* (Hildén *et al.*, 2007), *Dichomitus squalens* (Bak *et al.*, 2010), and *Pleurotus* spp (Fazaeli, 2007) and some degraders of the whole cell wall components have been identified such as *Heterobasidium annosum* (Daniel *et al.*, 1998), *Trametes versicolor* (Tanaka *et al.*, 1999), *P. chrysosporium* (Sánchez, 2009) and *Irpex lacteus* (Xu *et al.*, 2009a).

Various crop residues (wheat straw, madake bamboo, oil palm fronds, hays) have been degraded to a greater extent for animal use by some white rot fungi such as *Phanerochaeta chrysosporium, Ceriporiopsis subvermispora, Phlebia* sp., *Pleurotus* sp., *Coriolus versicolor*

(Kerem *et al.*, 1992; Barrasa *et al.*, 1995; Akin *et al.*, 1996; Adamović *et al.*, 1998; Arora and Sharma, 2009a; Shabtay *et al.*, 2009; Hassim *et al.*, 2012). Gupta *et al.* (1993) reported a higher degradation of lignocellulosic substrate percentages of 65 – 70% by *P. chrysosporium* and 45% by *Ganederma applanatum* and *Coriolus versicolor.*

1.2.14 *Factors influencing solid state fermentation*

Biological pre-treatment of substrates is usually done using a solid-state fermentation (SSF) process, where microorganisms are cultivated on a solid substrate with water available in minimal amount for supporting the microbial metabolism, as well as the growth of a particular fungus or mixed fungi (Reid, 1989), as against using the submerged fermentation (SmF) process. This is because SSF is more advantageous than SmF in that it aids in the reduction of mycotoxins, and produces higher protein enriched feed for animals as the process supports higher ligninolytic enzymatic function and stability needed for the substrate upgrade (Acuña-Argüelles *et al.*, 1995; Pandey, 2003; Hölker *et al.*, 2004). Due to its requirement for less enzyme sterility, it uses cheaper and smaller fermenters and substrates (Singhania *et al.*, 2009). It involves easy substrate disposal (Singhania *et al.*, 2009), high oxygen diffusion and solubility, less effluent production (Reid, 1989; Zhu *et al.*, 2011), less energy, and small waste water (Tengerdy, 1996) but produces valuable secondary metabolites (Durand, 2003).

During the SSF upgrade of a lignocellulosic substrate, fungi are able to break various complexes formed by lignin. Firstly they degrade lignin-hemicellulose complexes followed by lignin-cellulose complexes (Arora and Sharma, 2009a; Arora and Sharma, 2010; Xu *et al.*, 2010; Arora *et al.*, 2011; Tuyen *et al.*, 2012). This leads to higher digestibility of hemicellulose in most cases (Sharma and Arora, 2015).The process is usually carried out under a controlled environment, where several factors can be seen to influence the substrate fermentation. These factors include substrate (i.e. physical structure), fungal strains, fermentation or inoculation time, particle size, fermentation factors or culture conditions (i.e. temperature, pH, relative humidity), moisture content / water acidity, aeration, nitrogen and carbon supplementation, and inoculum quantity and type (Pandey, 2003; Krishna, 2005; Thomas *et al.*, 2013; van Kuijk *et al.*, 2015; Sindhu *et al.*, 2016; Sharma *et al.*, 2017). Some of these factors are discussed below.

1.2.14.1 *Substrate*

The type of substrate used is very essential in obtaining an efficient fermentation process when agro-industrial residues or by-products are used as major substrates. These residues are more lignified and fibrous and so are capable of supplying fungi with the required nutrients and

macromolecular matrix (i.e. cellulose, lignocellulose, starch, pectin, hemicellulose and other polysaccharides) from which the fungi obtain energy and carbon needed for their growth (Raimbault, 1998; Krishna, 2005; Bhargav *et al.*, 2008). The fungi tend to show higher preference for such substrates, that also increase their enzymatic functions (Bhargav *et al.*, 2008). Variation in the chemical composition of substrates has made comparison between pre-treatment researches difficult, as lignocellulosic substrates have been collected from different parts of various plant species and cultivars (Pérez *et al.*, 2002; Van Hulle *et al.*, 2010) or different regions and climatic zones (Arora and Sharma, 2009b) or have the same or different origins (Labuschagne *et al.*, 2000; Arora and Sharma, 2009b) and different batches of variable chemical compositions (Labuschagne *et al.*, 2000). Nutrient deficiencies limit substrate colonization by fungi. Straws can be classified into coarse (those with a tough outer cover) and slender (those with light outer cover) (Prasad *et al.*, 1993). The use of straws and other by – products as the degradation substrate varies depending upon the ability of a substrate to respond to a fungal treatment (Krishna, 2005). Straws with hard coating may not be highly digestible compared with those without hard covering. For instance, when *Dichomotus squalens*, *Pleurotus* spp., *Abortiporus bienni* were grown on beech sawdust, reed straw, rape straw and rice husk, the fungal growth was seen on various straws but not the rice husk (Henriksen *et al*., 1999). The obtained results can be attributed to the hard outer covering of the rice husk.

The use of substrate as a supporting medium is cheaper than the use of an inert material but, as fermentation proceeds, the substrate loses its rigidity and structure due to its degradation, which affects the transfer of heat and mass in the system (Krishna, 2005). The efficiency of fungal degradation of the substrate can be increased if the substrate is pre-treated (i.e. physical, chemical and physicochemical) and/or supplemented with nutrients that are lacking in the substrates. The pre-treatment exposes more sites on the substrates for further hydrolysis while nutrient supplementation is needed for proper fungal growth and colonisation. Most straws exhibit some attributes, such as silification, lignification, cellulose crystallinity, encrustation, etc., which negatively influence the digestion of cellulose and hemicellulose (Mahesh and Mohini, 2013). For instance, a higher degree of crystallinity reduces the rate at which the straw is degraded by cellulolytic microbes (Pérez *et al.*, 2002; Mahesh and Mohini, 2013). Also, lignin constituents, i.e. guaiacyl propanol, syringyl alcohol and p-hydroxy phenyl propanol, respond differently to fungal degradation where syringyl-units tend to degrade earlier than the guaiacyl units (van Kuijk *et al.*, 2015) read more in Table 1.

1.2.14.2 *Fungal strain*

Variations in lignin degradation of a particular substrate have been recorded for different fungal types, i.e. brown, soft and white rot fungi (Abdel-Azim *et al.*, 2011; del Vecchio *et al.*, 2012; van Kuijk *et al.*, 2015; Daniel, 2016; García-Torreiro *et al.*, 2016), fungal species (Capelari and Zadrazil, 1997; Arora and Sharma, 2009b; Arora *et al.*, 2011; Sebata *et al.*, 2011; Shrivastava *et al.*, 2011; Cone *et al.*, 2012; Tuyen *et al.*, 2012; Tuyen *et al.*, 2013), and fungal strains (Fazaeli, 2007; Membrillo *et al.*, 2008; Akinfemi, 2010a). The *Pleurotus* strains exhibited different ligninolytic enzymatic function when grown under the same conditions on sugarcane bagasse (Membrillo *et al.*, 2008), grapevine sawdust (Stajić *et al.*, 2006a), and agricultural wastes, i.e. grapevine sawdust and mandarine peels (Stajić *et al.*, 2006b) respectively. The identification and screening of the best or superior fungal strains for lignin degradation can be used for breeding programmes related to the production of biologically improved feed (Lettera *et al.*, 2011; del Vecchio *et al.*, 2012), thus serving as an alternative feed for better ruminant production that actually utilises structural carbohydrates (read more in Table 1).

1.2.14.3 *Fermentation or inoculation time*

Biological (i.e. fungal) pre-treatment of substrates that are high in lignocellulose usually takes a longer period before efficient delignification can be recorded compared to other pre-treatment methods, as the fungi need to undergo several stages of growth to degrade the substrates that are usually recalcitrant in nature (Zhong *et al.*, 2011; Cone *et al.*, 2012; Tuyen *et al.*, 2012; Sindhu *et al.*, 2016; Sharma *et al.*, 2017). The time also varies with the type of substrate and fungi used for the pre-treatment, e.g. efficient degradation of lignin was recorded on the third week when grass was pre-treated with *P. chrysosporium* (Liong *et al.*, 2012). The stages involved in the fungal pre-treatment of lignocellulosic substrates include the colonizing stage, fruiting stage and the mature stage and various activities occur at a different point in time during these stages.

The colonizing stage includes the primary and secondary metabolic stages. At the primary metabolic stage, the fungi convert a digestible portion of the substrate into sugar for their establishment on substrates (Karunanandaa and Varga, 1996). If substrates are used for animals at this stage, available nutrients for use by rumen microbes are limited because the nutrients are used up by the microbes for their initial growth (Sharma and Arora, 2015). On the other hand, at the secondary metabolic stage the soluble fraction would have been utilized by the fungi which degrade lignin and other structural carbohydrates through enzymatic functions (Rolz *et al.*, 1986; Agosin *et al.*, 1987; Eriksson, 1988; Karunanandaa *et al.*, 1995). This stage seems to

be the best for the fungal treatment of substrates, as more soluble fractions are available for animal use. At the fruiting stage, unusual amounts of available soluble carbohydrates are utilised as carbon or energy sources in preparation for fruit body production (Jalč, 2002). This stage should be prevented in fungal-substrate upgrade as it reduces the amount of carbohydrates available to animals, and the time used for colonising is affected by the type and quantity of an inoculum.

At the mature stage, the fungi are fully grown, and they begin to digest themselves or destroy their cells by their enzymes (Autolysis). This stage is accompanied with a higher concentration of minerals and digestibility is reduced (Karunanandaa *et al.*, 1995). For instance, growing *Pleurotus ostreatus* on wheat straw after the seeding stage led to an insignificant effect on lignin degradation. However, this was accompanied with a decrease in NDF and ADF contents of the straw (Adamović *et al.*, 1998). Therefore, the optimal incubation time needs to be determined to prevent fungal consumption of soluble carbohydrates in the process of trying to achieve lignin degradation in substrates as a possible improved animal feed (Du *et al.*, 2011; Sindhu *et al.*, 2016; Sharma *et al.*, 2017); read more in Table 1.

1.2.14.4 *Particle size*

The growth of fungi and product formed is affected by the particle size of the substrate. A small particle sized substrate supports the growth of fungi better than a substrate with a larger size. This is because it provides an adequate surface area required for fungal activity. The use of too small particle sized substrate prevents air transfer in the substrate, which limits the fungal growth (i.e. agglomeration). Also, the use of large or coarse particle-size substrate does not provide an adequate surface area for fungal activity, but air transfer in the substrate is well supported (Pandey *et al.*, 1999; van Kuijk *et al.*, 2015; van Kuijk *et al.*, 2016b). To obtain better degradation by fungi, the substrate particle size should be large enough to facilitate aeration and be small enough (as discussed further in Table 1) to provide adequate surface area required for fungal activity. To reduce the coarse nature of various substrate, methods such as grinding, chipping or chopping can be used (read more in Table 1).

1.2.14.5 *Temperature*

The spore formation and growth of the microorganism, as well as the products of fermentation (enzymes and metabolites), are affected by the fermentation temperature. A wide range of temperature is possible for the growth of fungi, but to achieve a better formation of products by each fungus an optimum temperature is needed (Bhargav *et al.*, 2008; van Kuijk *et al.*, 2015). Also, increased speed and sequence of substrate decomposition is dependent on the

fermentation temperature. The temperature range between 25^0-30^0C tends to favour better speed of substrate decomposition by most fungi (Sindhu *et al.*, 2016), which can grow between the temperature range of 20^0-55^0C (Krishna, 2005).

In SSF, temperature significantly influences the microbial growth and function and it can cause inhibition of enzymes and metabolites, protein denaturation, and cell death (Gowthaman *et al.*, 2001; Krishna, 2005). In an experiment carried out by Zadrazil (2000), the rate and sequence of substrate decomposition were significantly influenced as the temperature increased from 22^0C to 30^0C. Also, in the production of cellulose, the enzyme (β-glucosidase and endo-glucanase) activity was inhibited as the incubation temperature was increased (Sohail *et al.*, 2009). In SSF, the growth of fungi on substrates led to heat generation, as obtained from the metabolic activities of the fungi. This heat continues to accumulate in the system because of the lower thermal conductivities that exist in the substrates. As a result of heat accumulation, the product required becomes denatured. At this stage oxygen is limited, which is a fundamental requirement for better product formation by the fungi (Pandey, 2003); read more in Table 1.

1.2.14.6 *Moisture content and Water activity (Aw)*

In SSF, the roles played by water are usually observed at two levels (i.e. cellular and molecular). At the cellular level, it provides cells with nutrients, removes metabolites and waste and maintains cell stability and membrane permeability. At the molecular level, biopolymer cells, nucleotides and proteins are stabilised (Quinn, 1985). In the absence of sufficient water in a system, the cellular metabolism is slowed down due to the accumulation of secondary metabolites, inappropriate gas and lack of nutrients. Lack of water generally obstructs the fungal metabolic pathway by not maintaining the enzyme's functional properties. Physicochemical factors, e.g. temperature and aeration, are influenced by the transfer of water mass (Gervais and Molin, 2003). In SSF, when low moisture content exists within the growing system, it limits the microbial metabolism and growth, the stability of an enzyme, nutrient diffusion and substrate swelling (Lonsane *et al.*, 1992). High moisture content can limit gas transfer which can lead to particle agglomeration (Gowthaman *et al.*, 2001).

The interaction existing between water and solids as well as other non-aqueous constituents is referred to as water activity (Bhargav *et al.*, 2008). The investigation carried out on the effect of Aw on the growth of hyphae, metabolite production, fungal physiology and orientation revealed that the rate of radial extension of *P. roqueforti* and *I. viride* was significantly affected when Aw was reduced. The optimal Aw of 0.97 and 0.99 has been recorded for *P. roqueforti* and *I. viride* respectively, whereas values below 0.90 for the two fungi revealed no radial

extension by the fungi (Gervais and Molin, 2003). Water activity tends to affect fungal germination, fungal growth rate by elongating the fungal lag phase thus lowering its biomass production, the availability of nitrogen and carbon in the system, as well as formation of secondary metabolites (Grajek and Gervais, 1987; Oriol *et al.*, 1988; Passamani *et al.*, 2014). Fungal growth is greatly influenced by the amount of water to air ratio in the substrate. The addition of water to a constant weight of substrate makes the substrate devoid of air, therefore making the substrate swell up and creating an unfavourable growth environment. All fungi require a particular range of water content, usually between 25 – 150ml / 25 g of the substrate, and water contents below or above this range decrease the degradation rate of the substrate (Zadražil and Brunnert, 1981).

1.2.14.7 *pH*

The formation of secondary metabolites during fungal metabolism requires favourable growth conditions. To obtain this, controlling the pH is very vital in any fermentation process and fungal cultivation on substrates (Sindhu *et al.*, 2016). In SSF, reduction in culture pH is usually caused by organic acid (i.e. acetic, citric and lactic acids) being released during fungal metabolism. Also, the use of urea can increase the culture pH while hydrolysis of urea can lead to assimilation of organic acids and alkalinisation of the system (Raimbault, 1998; Krishna, 2005). The pH range of 3.8 – 6.0 is considered optimal for the formation of metabolites and fungal growth, although most filamentous fungi have been found to be growing on a broad range of pH between 2- 9, but not entirely exhibiting their activity (Gowthaman *et al.*, 2001). During substrate formulation, consideration of the components buffering capacity or ensuring that the chemical use during the formulation does not biologically affect the fungal activity is essential in controlling the pH in SSF (Krishna, 2005; van Kuijk *et al.*, 2015).

1.2.14.8 *Aeration*

Removal of heat, CO_2, volatile metabolites and moisture is achieved by ensuring oxygen is available in the culturing system. Aeration is required for fungal growth most especially in the SSF method (Krishna, 2005). The rate of transfer of oxygen in the culturing system is influenced by reactor configuration parameters such as substrate porosity, moisture content, substrate size, the speed of drum rotation and reactor depth (Gowthaman *et al.*, 2001; Krishna, 2005). In tray fermentation experiments, keeping the reactor bed depth at minimum level facilitated aeration and reduced heat-gradient formation. In an experiment carried out by Mitchell *et al.* (1990) using the SSF method, the effect of oxygen on *Rhizopus oligosporus*

growth was examined. It was observed that wet fungal layer thickness and surface (i.e. interfacial gas-liquid surface area) influenced the rate of oxygen transfer.

Furthermore, the concentration of carbon-dioxide and oxygen in the growing media influences the lignin degradation and organic matter losses. In an experiment where wheat straw was treated with *Pleurotus sajor – caju* (Kamra and Zadražil, 1986), the fermentation process at 100% atmospheric oxygen led to high OM loss and high *in vitro* digestibility while lignin degradation was slowed down at rates below 20% atmospheric oxygen. The fermentation process occurring between 1-20% carbon-dioxide (CO_2) in the atmosphere did not affect organic matter and lignin losses, above 30% there was a slight increase in organic matter and lignin losses. Better digestibility was observed at 1 -10% of CO_2 while higher percentages lead to reduced digestibility.

1.2.14.9 *Nitrogen and carbon supplementation*

The addition of inorganic nitrogen sources (e.g. ammonium nitrate, NH_4NO_3, peptone, and malt extract) and carbon sources (e.g. glucose, molasses) to substrate treated with fungi induces higher colonization, thus facilitating the rate and sequence of substrate degradation by fungi (Gowthaman *et al.*, 2001; Sharma and Arora, 2010b; Wan and Li, 2011; Lv *et al.*, 2014; Rouches *et al.*, 2016). The concentrations of the nitrogen source also influence degradation. In an experiment carried out by Zadražil and Brunnert (1982b), the addition of NH_4NO_3 at lower level facilitated the rate of degradation of the substrate by *Stropharia rugosoannulata*, *Agrocybe aegerita* and *Pleurotus florida,* while *Pleurotus eryngii* degradation activity was inhibited therefore having no effect on lignin component of the substrate. However, higher concentrations of NH_4NO_3 led to a decreased rate of substrate degradation by all the fungi. In SSF process, lignin degradation by fungi does take place in the absence of nitrogen in the substrates in most cases, as excess nitrogen inhibits fungal lignolytic function (van Kuijk *et al.*, 2015). The use of nitrogen supplements is needed in animal feeds to improve the palatability and nutritive value of the feed. However, this should be considered after fungal treatment and before feeding the SSF to an animal if it has a negative effect on the enzymatic function of the fungi. Fungal pre-treated forages are usually not palatable (Villas-Bôas *et al.*, 2002) and this does negatively affect the voluntary intake, so there is the need to look into improving the voluntary intake of SSF based diets (Mahesh and Mohini, 2013; van Kuijk *et al.*, 2015).

1.2.14.10 *Inoculum type and quantity*

This usually affects the time at which the substrate is colonized by the microbe, a smaller quantity of inoculum leads to a longer time of substrate colonization, while the use of a larger

quantity of inoculum gives the opposite. On the other hand, the quantity of inoculum does not have an increasing effect on the fungal enzymatic function involved in lignin degradation, i.e. more quantity of inoculum does not mean that it will lead to an increased enzyme production (Mehboob *et al.*, 2011). Fungal colonization of substrate requires an inoculum unit, and this can either be a spore (i.e. the smallest inoculum unit), a spawn/mycelium coated grain (i.e. the largest inoculum unit), and/or small pieces of initially treated substrate. The spore is usually used on a small scale basis, and it can either be a vegetative or generative spore but vegetative spores are most preferred (Saxena *et al.*, 2001; van Kuijk *et al.*, 2015; van Kuijk *et al.*, 2016b). The spawn is used on a large scale basis for mushroom production (Tripathi and Yadav, 1992), but since they are usually larger than spores they tend to be limited in that they result in fewer sites being inoculated when presented with the same quantity of inoculum as the spores over a particular period (van Kuijk *et al.*, 2015). The use of a fungal treated substrate is simple and more accessible but it is limited in that, when consistently used, it leads to fungal degeneration therefore not breeding true to the original strain due to genome instability (van Kuijk *et al.*, 2015); read more in Table 1.

Table 1 Various fermentation factors used in different lignin degradation studies using different *Pleurotus* species and *Physisporinus rivulosus* (*P. rivulosus*, other name is *Ceriporiopsis rivulosus*)

Reference	Fungal species	Tempt / time	Substrate and its size	Inoculum
Shrivastava *et al.* (2011)	*P. ostreatus* F6 (Jaquin ex. Fr) kammer	30^0C/ 30days	Wheat straw / 1.5-2.0cm	0.5% w/v of fungal dry weight / wheat straw
Fazaeli (2007)	*P. ostreatus* *P. florida*	22 ± 5^0C/49 days	Wheat straw	3.5kg of mycelium suspension in 100kg of straw
Adamović *et al.* (1998)	*P. ostreatus*	24^0C/45days	Wheat straw/3-4cm	grain spawn at 2% of substrate fresh weight
Akinfemi (2010b)	*P. ostreatus* *P. pulmonarius*	30^0C/ 21days	Maize cob/1mm	2pcs of 10mm mycelial disc / 25g cob
Hakala *et al.* (2005)	*P.. rivulosus* T241i	28^0C/14days	Norway spruce (*P. abies*) wood chips/ 3mm x 20mm x 30mm	150ml of homogenised mycelium/300g dry weight of substrate
Hildén *et al.* (2007)	*P. rivulosus* T241i	28^0C/35days	Norway spruce sawdust Charcoal/ 1-2mm	4% v/v of homogenised mycelium/substrate
Maijala *et al.* (2008)	*P. rivulosus* T241i	25^0C/42days	Scots pine wood (P. sylvestris) Norway spruce wood (P. abies)	wood blocks soaked in homogenised mycelium suspension for 2min under vacuum
Labuschagne *et al.* (2000)	*P. ostreatus* (Jacq. ex Fr.) kummer	22-24^0C/21days	Wheat straw/ small pieces	4% grain spawn/ 10 kg of moist wheat straw
Tuyen *et al.* (2012)	*P. ostreatus* MES 03449 *P. eryngi* MES 03757	24^0C/49days	Wheat straw/2-3cm	5g of grain spawn/160g of moistened wheat straw
Tuyen *et al.* (2013)	*P. ostreatus* MES 03449 *P. eryngi* MES 03757	24^0C/42days	Rice straw & maize stover (2-5cm)	5g of grain spawn/160g of moistened substrate

1.2.15 *Effect of aerobic fungal treated straw on nutrient utilisation, growth and production of ruminant animals*

Research involving the use of fungal treated straw in feeding ruminant animals has led to an increased utilization of nutrients, balanced nitrogen requirement and increased body weight (Omer *et al.*, 2012; Shrivastava *et al.*, 2012; Mahesh and Mohini, 2013). However, the consistency of these positive results obtained was dependent on the type of WRF used.

The inclusion of these treated straws in the feed of ruminants at various percentages was found either not to have an effect, or it reduced the DM intake and digestibility due to the high content of ash produced by the fungal degradation (Jalč *et al.*, 1994; Capelari and Zadrazil, 1997; Sharma and Arora, 2015)

Supplementation of fungal treated straws with groundnut cake or urea led to an increased intake, digestibility and nitrogen retention, which was found to be higher in cross-bred calves supplemented with groundnut than with urea (Wallis *et al*., 1988). Feeding of Simmental heifers with spent compost from edible *P. ostreatus* at above 17% level of inclusion in the diet reduced their weight gain significantly (Adamović *et al.*, 1998). However, feeding Pelibuey sheep with spent maize straw from edible *P. ostreatus* increased the feed intake and improved body weight gain of these sheep (Díaz and C. Sánchez, 2002).

Feeding of cattle with wheat straw (Fazaeli *et al.*, 2002) treated with fungi increased the intake and digestibility (organic matter and dry matter) by over 10 %. Replacement of wheat offal with fungal processed maize cobs in a guinea grass based diet fed to West African Dwarf lambs led to higher growth rate and dry matter intake (Akinfemi and Ladipo, 2011). Feeding goats with wheat straw treated with *Ganoderma* sp. rckk02 (WRF) led to an increased DM intake and digestible crude protein by the animals (Shrivastava *et al.*, 2012).

When ligninolytic substrates were upgraded by WRF to protein – rich substrates, the digestibility of the upgraded product was low when compared with the un-upgraded substrates (Villas-Bôas *et al.*, 2002) which may be due to reduced palatability. It was evident that the spent substrates obtained from fungal treatment were not very tasty and they required adding or mixing with more palatable ingredients to improve their intake and digestibility (Mahesh and Mohini, 2013). Gupta *et al.* (1993) combined the treatment of a substrate with both urea and WRF (*Coprinus fimetarius*) over a period of 35 days in an unsterilized environment. The substrate was firstly treated with urea for 30 days after which the fungus was used on the same substrate for the next 5 days. This increased the CP content but contributed to a higher loss of dry matter during the fungal treatment.

Lactating cows require some quantity of NDF sourced from high-quality forage to facilitate the growth of rumen microbes, therefore maximizing their function in the rumen which leads to better milk production. However, an insignificant effect was observed on daily intake, digestibility and daily milk production when fungal treated wheat straw was fed to lactating Holstein cows. The fungal treated wheat straw was included at different levels of 0, 10, 20 and 30% in the diet as a replacement for alfalfa hay, which showed a better weight gain of those cows (Fazaeli *et al.*, 2002).

1.2.16 *Effects of aerobic fungal treatments on in vitro fermentation parameters, total gas and methane production*

In the rumen, gas produced during fermentation is regarded as a loss of nutrients that were supposed to be utilised by the animals. However, during *in vitro* gas production, the gas measurements are the reflection of the rate and extent at which feeds were degraded (Blümmel *et al.*, 1997). During *in vitro* gas production experiments using fungal treated substrates, the three following factors influence the amount of gas produced by these substrates (Gupta *et al.*, 1992; Cone *et al.*, 1997; Cone and Becker, 2012): i) The upgraded easily digestible portion of the substrate (i.e. the soluble carbohydrate), ii) The non-improved portion (i.e. the fibrous plant cell wall), iii) The increased microbial number caused by the upgrading (Chumpawadee *et al.*, 2007) leads to high percentage of gas and short chain fatty acids.

Findings from several researchers, such as Okano *et al.* (2005); Suzuki *et al.* (1995); Akinfemi (2010b); Valizadeh *et al.* (2008); van Kuijk *et al.* (2016a); and Zuo *et al.* (2018), confirmed the above-listed facts when *in vitro* studies were carried out using fungal treated straws. However, Kaur *et al.* (2010) discovered the opposite in an *in vitro* experiment when fungal treated wheat straw and rice straw were used at a ratio of 1:1. Fungal-treated straws recorded reduced TGP, and insignificant ammonia nitrogen (NH3-N) when compared to the untreated straws. Experiments carried out by Karunanandaa and Varga (1996); Suzuki *et al.* (1995) and Salman *et al.* (2008) discovered an increase in the amount of total volatile fatty acids and NH_3-N. In addition to this, an increased pH was obtained (Abo-Donia *et al.*, 2005; Omer *et al.*, 2012). Tripathi *et al.* (2008) discovered in their *in vitro* experiments that fungal treated straw reduced the population of large holotrichs while it increased the population of small holotrichs.

Fungal treated substrates are mostly seen as an upgraded feed because they provide rumen microbes (i.e. fungi and fibre digesting bacteria) with a larger surface areas for their increased activity. This reduces the amount of time the substrates spend in the rumen and the amount of methane gas produced (Moss *et al.*, 1994; Sallam *et al.*, 2007; Mahesh and Mohini, 2013). Higher rumen methane is produced majorly when fibrous feeds are fed to animals. This is

because microbes spend a considerable amount of time while trying to degrade and ferment the substrates in the rumen (Torrent *et al.*, 1994; Johnson and Johnson, 1995; Boadi *et al.*, 2004). Several researchers, such as Jalč *et al.* (1994) and Akinfemi (2010b), recorded reduced methane production in their *in vitro* studies using fungal treated straws or crop residues.

1.2.17 *Effect of anaerobic fungal treated straw on nutrient utilisation, growth and production of ruminant animals (in vivo studies)*

A positive relationship has been established between the presence of anaerobic fungi in the rumen and voluntary feed intake, as well as digestibility, when low-quality forages are made available to ruminant animals (Lee *et al.*, 2000a; Thareja *et al.*, 2006). This is because these fungi preferentially colonize and degrade structural carbohydrates for the release of simple sugars, which is achieved through their rhizoids penetrating the macro-structure of the forage and the release of extracellular enzymes such as cellulases and hemicellulases (Nagpal *et al.*, 2009; Sirohi *et al.*, 2013), thus increasing intake and digestibility (Theodorou *et al.*, 1990; Shelke *et al.*, 2009). The degradation of structure also aids in exposing more sites for further hydrolysis by other rumen microbes, especially bacteria, therefore facilitating better digestibility. Also, several researchers have investigated inoculating low-quality feeds with ruminal fungi as additives in ruminant feeds, and their use has been seen as a possible means of increasing ruminant productivity and performance (read more details in Table 2).

1.2.18 *Effects of anaerobic fungal treatments on in vitro and in sacco nutrient digestibility, fermentation parameters, pH, total gas, and methane production as well as ensiled substrates.*

Findings from several researchers have identified that the use of anaerobic fungi in *in vitro* and *in sacco* studies increased nutrient degradability and or digestibility (Elliott *et al.*, 1987; Lee *et al.*, 2000a; Kumar *et al.*, 2004; Thareja *et al.*, 2006; Dayananda *et al.*, 2007; Nagpal *et al.*, 2009; Shelke *et al.*, 2009; Paul *et al.*, 2010; Nagpal *et al.*, 2011), increased ammonia nitrogen (Lee *et al.*, 2000a), increased TVFAs with a proportionally larger increase in acetate than in propionate (Elliott *et al.*, 1987; Lee *et al.*, 2000a; Paul *et al.*, 2010; Nagpal *et al.*, 2011; Sirohi *et al.*, 2013); increased gas volume (Paul *et al.*, 2010; Sirohi *et al.*, 2013) and increased pH (Elliott *et al.*, 1987; Lee *et al.*, 2000a). These fungi affected the fermentation parameters as well as nutrient degradability due to their ability to increase total viable bacterial count as well as cellulolytic bacterial (Lee *et al.*, 2000a) and fungal counts (Lee *et al.*, 2000a). Also, the increase in cellulolytic microbes was accompanied by a higher enzymatic activity (Thareja *et al.*, 2006; Nagpal *et al.*, 2009; Nagpal *et al.*, 2011; Sirohi *et al.*, 2013) that led to further increase in nutrient digestibility and or degradability (read more details in Table 3 and 4)

On the other hand, some researchers recorded an insignificant or reductive effect on TVFA content, especially propionate and butyrate levels, and nutrient digestibility (Samanta *et al.*, 2001; Paul *et al.*, 2004; Paul *et al.*, 2006; Shelke *et al.*, 2009). Anaerobic fungal fermentation of substrates in the rumen leads to the production of H_2 (Mountfort and Orpin, 1994), a primary substrate for methane production. Therefore, there is the possibility that an increase in the ruminal fungal population might lead to an increase in methane production. Paul *et al.* (2010) recorded an increase in methane production with the addition of fungal (*Piromyces* sp.) cultures on in vitro fermentation of wheat straw.

Lee *et al.* (2015) investigated the use of three species of anaerobic fungi (*Piromyces*, *Orpinomyces* and *Neocallimastix*) as inoculants of rice straw ensiled for 120 days. Within 30 days the fungi reduced the pH to very low values. The fungal population increased from day 10 to day 30 after which there was no further growth. As a result of the proteolytic activity of the fungi, the CP content as well as the ammonia nitrogen increased from 10- 60 days, while at the same time the fibre content decreased due to their cellulolytic activity, and dry matter degradability increased.

Table 2 Summarised methods and findings of past research that used anaerobic rumen fungi for *in vivo* studies

Researchers	Topic	Methodology	Findings
Sehgal *et al.* (2008)	Influence of anaerobic fungal administration on growth, rumen fermentation, and nutrient digestion in female buffalo calves	*Neocallimastix* sp. GR1 was isolated from goat and was administered orally by drenching 250ml (≈10^6tfu/ml) of the fungal isolate every 4th day to female buffalo calves (5-8 months of age) consuming a total mix ration (wheat straw + green oats + concentrates. Animals not receiving the fungal isolate were used as control.	The values ranged from the control to fungal treated animal. • Similar DMI (4.14 -4.12 g/d). • DCP (275.13 – 295.39 g/d) • Improved weight gain (520.18 – 659.81 g/d). • Increased TDN intake (2.19 – 2.46 kg/day). • Increased feed efficiency (12.58-16.19%). • Increased coefficients of apparent digestibility of OM, DM, CP, CF, NDF, ADF, Cellulose, DCP and TDN of the substrate. • Decreased pH (7.16 – 6.99). • Increased TVFA mM/100ml (10.29-13.37). • Increased total nitrogen mg/100ml (77.39 – 105.47). • Decreased ammonia nitrogen mg/100ml (13.3 – 8.71). • Increased TCA precipitable nitrogen mg/ 100ml (52.69 – 71.07). • Increased fungal zoospores (1.38 – 3.83 x 10^5 /ml).
Theodorou *et al.* (1990)	The effect of a fungal probiotic on intake and performance of early weaned calves	*Neocallimastix* sp., R1 was isolated from sheep and was administered orally through drenching every day to early weaned Fresian bull calves. Animals not receiving the fungal isolate were provided as control.	The values ranged from the control to fungal treated animal. • Increased DMI (1.35 – 1.85 g/d). • Increased weight gain (700 – 800 g/d)
Dey *et al.* (2004)	Influence of an anaerobic fungal culture (*Orpinomyces* sp) administration on growth rate, ruminal fermentation and nutrient digestion in calves	*Orpinomyces* sp., C-14 was isolated from cows and was administered orally through drenching 160 ml (≈10^6tfu/ml) every week to cross bred calves (Tharparkar x Holstein-Fresian (10 months) feeding on TMR (Wheat straw and green oats).	The values ranged from the control to fungal treated animal. • Increased weight gain (614.8 – 709 g/d). • Similar DMI (366.78 – 363.79 g/d). • Increased DCP (368.65 – 394.18 g/d). • Increased TDN intake (2.16 – 2.46 kg/day). • Increased coefficients of apparent digestibility of DM, CP, CF, NDF, ADF, and Cellulose. • Decreased pH (7.18 – 7.01). • Increased TVFA mM/100ml (11.57 – 13.03).

		Animals not receiving the fungal isolate were used as control.	• Increased total nitrogen mg/100ml (84.00 – 109.20). • Decreased ammonia nitrogen mg/100ml (15.52–7.93). • Increased TCA precipitable nitrogen mg/ 100ml (57.5-87.97). • Increased fungal zoospores (1.08 – 2.42 x 10^5).
Saxena *et al.* (2010)	Effect of administration of rumen fungi on production and performance of lactating buffaloes	*Orpinomyces* sp., C-14 and *Piromyces* sp., WNG-12, were isolated from cattle and wild blue bull respectively. 250 ml (3 – 5 days of growth) of the isolate was administered orally through drenching every week to lactating buffaloes feeding on TMR (wheat straw + concentrate + green maize). Animals not receiving the fungal isolate were used as control.	The values ranged from the control to *Orpinomyces*; and *Piromyces* treated animals. • Increased milk yield kg/d (8.03 – 8.42; 8.46). • Similar DMI kg/d (11.79 – 11.50; 10.62). • Improved feed efficiency kg milk/100kg DM (67.14 – 72.99; 81.13). • Increased coefficients of apparent digestibility of OM, DM, CP, NDF, ADF, Cellulose, DCP, and TDN with *Piromyces* sp recording higher values than *Orpinomyces* sp. • Decreased pH (7.04- 6.96; 6.90). • Increased TVFA mM/100ml (6.27- 10.89; 11.45). • Increased total nitrogen mg/100ml (74.29- 95.57; 105.84). • Decreased ammonia nitrogen mg/100ml (14.84 – 7.70; 6.53). • Increased TCA precipitable nitrogen mg/ 100ml (52.45-80.08; 89.41). • Increased fungal zoospores (1.66 – 2.59 x 10^5; 2.87 x 10^5).

Table 3 Summarised methods and findings of the past research that used anaerobic rumen fungi for *in sacco* studies

References	Topic	Methods	Findings
Lee *et al*. (2000a)	Influence of an anaerobic fungal culture administration on *in vivo* ruminal fermentation and nutrient digestion	*Orpinomyces* sp., KNGF-2 was isolated from Korean native black goat. 200ml of 7 day culture was administered intra-ruminally through cannula to Crossbred sheep (Corriedale x Polworth) feeding on Orchad grass hay (560g) + concentrate (240g). Animals not receiving the fungal isolate were used as control	The values ranged from the control to fungal treated animal. • Increased pH (6.15 – 7.00). • Increased ammonia nitrogen mg/l (12.28 – 14.20) at 6h post feeding. • Increased calculated effective degradation (32.2 – 33.0). • Increased coefficients of apparent digestibility of DM, CP, NDF, ADF, Cellulose and hemicellulose of the substrate. • Increased TVFAs, acetate, propionate and butyrate concentrations from 0h–9h respectively with the 3h post feeding recording the highest TVFA (72.9-113.1mM); acetate (48.2- 61.8 mM); and propionate (11.4 -33.2 mM) while 6h post feeding recorded the highest butyrate concentration (9.0 -14.7 mM) similar to 3h. **After 6h post feeding:** • Increased total viable bacteria (10.47 – 21.95 x 10^9 cfu/ml). • Increased cellulolytic bacterial count (15.5 – 19.5 x 10^6 / ml). • Increased fungal count (4.23 – 8.62 x 10^4 tfu / ml).
Elliott *et al*. (1987)	The influence of anaerobic fungi on rumen VFA concentration *in vivo*	*Neocallimastix* sp. A1 was isolated, 200 ml was administered intra-ruminally through cannula to cannulated Merino cross bred sheep fed chaffted barley straw + sunflower meal.). Animals not receiving the fungal isolate were used as control	The values ranged from the control to fungal treated animal. • Increased pH (6.8 – 7.0). • Increased DM loss % (44.6 – 53.8) after 48h. • Increased DMD % (46.2 – 49.5). • Increased acetate concentration mM/100ml from 0.63 (0h) – 0.77 (72h). • Decreased propionate concentration mM/100ml from 0.29 (0h) – 0.15 (72h). • Similar butyrate concentration from 0h to 72h.

Table 4 Summarised methods and findings of the past research that used anaerobic rumen fungi in *invitro* studies

References	Topic	Methods	Findings
Dayananda *et al.* (2007)	Biodegradation of urea-ammonia treated wheat straw using anaerobic rumen fungi	*Orpinomyces* sp., C-14 and *Piromyces* sp., WNG-12 were used. 5ml ($\approx 10^6$ cfu/ml) of each fungus was used along with buffered inoculum to incubate Urea-NH_3 treated wheat straw for 48 and 72h respectively.	The values ranged from the control to *Orpinomyces*; and *Piromyces* incubated substrates respectively: • Increased IVDMD % (44.5 – 53; **55)** after 48h; (45.5 – 54.3; **55.8) after** 72h. • Decreased NDF % (78 – 76; **75**) after 48h; (62 – 60; **55**) after 72h. • Decreased ADF % (57 – 36; **34**) after 48h; (48 – 25; **23**) after 72h. • Decreased ADL % (8.2 - 7.5; **6.8**) after 48h; (8 – 6.5; **5.8**) after 72h
Paul *et al.* (2004)	Effect of anaerobic fungi on *in vitro* feed digestion by mixed rumen microflora of buffalo	*Piromyces* sp., FNG5 (isolated from nilgai); *Anaeromyces* sp., FBB1 (isolated from blackbuck); *Orpinomyces* sp., FS1 (isolated from sheep); *Piromyces* sp., FHD1 (isolated from hogdeer); *Piromyces* sp., FSD4 were used. 2.5 ml ($\approx 10^4$ cfu/ml) of each fungus were used along with buffered inoculum to incubate wheat straw +wheat bran (80:20) mix for 24 and 48h respectively. Control i.e. substrates without fungi were provided.	The values ranged from the control to FNG5; FBB1; FS1; FHD1; and FSD4 incubated substrates respectively: • Inconsistent apparent digestibility % (28.61–**35.31**; 28; 27; 32; & 31) after 24h; (41-41; 39; 38; 41; & **42**) after 48h. • Inconsistent true digestibility % (35.37-**43.64**; 35; 34; 40; & 39) after 24h; (49-51; 49; 48; 51; **52**) after 48h. • Inconsistent NDF digestibility % (18.47-**29.30;** 18; 16; 24; & 23) after 24h; (37-39; 36.5; 35; 38; & **39.5**) after 48h. • Inconsistent TVFAs concentrations mmol/100ml (5.68 – **6.35**; 5.25; 5.74; 6.02; & 5.42) after 24h; (8.24 – 7.73; 7.79; 7.49; **8.36; &** 7.80) after 48h. • Inconsistent acetate concentration mmol/100ml (3.86 - **4.39**; 3.54; 3.99; 3.90; & 3.74) after 24h; (5.81 – **5.30;** 5.20; 5.04; 5.23; & 5.27) after 48h. • Inconsistent propionate concentration mmol/100ml (1.0-**1.14**; 0.85; 0.95; 1.10; & 0.97) after 24h; (1.34 – 1.36; 1.42; 1.23; **1.59**; & 1.39) after 48h. • Inconsistent butyrate concentration mmol/100ml (0.49 –0.48; 0.61; 0.59; **0.75;** & 0.46) after 24h; (0.64 – 0.75; **0.82;** 0.81; 1.0; & 0.75) after 48h. • Increased CMCase, xylanase, acetyl esterase, and β-glucosidase activity was recorded at 24 & 48h. FNG5 recorded the highest enzymes activity.

Nagpal *et al.* (2009)	*In vitro* fibrolytic activity of the anaerobic fungus, *Caecomyces* sp., immobilised in alginate beads	*Caecomyces* (free moving) and *Caecomyces* (Immobilised with alginate beads) were used. 5.0ml (≈10^6 cfu/ml) of each fungus was used along with buffered inoculum to incubate wheat straw (500mg) for 48h and 72h respectively. Control i.e. substrates without fungi were provided.	The values ranged from the control to *Caecomyces* free moving: & immobilised incubated substrates respectively: • Increased IVDMD % (38.4 - **43.2**; & 39.9) after 48h; (38.7- **46.8**; & 45.1) after 72h. • Increased CMCase, xylanase, and FPase activity was recorded with free moving *Caecomyces* recording higher values (14.2; 22.8; 17.7 mIU/ ml) respectively than immobilised *Caecomyces*.
Lee *et al.* (2000a)	Influence of an anaerobic fungal culture administration on *in vivo* ruminal fermentation and nutrient digestion	*Orpinomyces* sp., KNGF-2 isolated from Korean native black goat was given to animal and the rumen fluid was collected for in vitro experiment. The rumen fluid was used in preparing buffered inoculum that was used to incubate 250mg of rice straw for 48h and 72h respectively. Control (i.e. substrates not treated)	The values ranged from the control to treated substrates: • It increased the IVDMD % (31.8 – 34.00) after 48h; **(33.2 – 41.8)** after 72h. • Increased cellulose and xylanase activity was recorded. 6h post feeding gave higher activity than 9h post feeding.
Thareja *et al.* (2006)	*In vitro* degradation of wheat straw by anaerobic fungi from small ruminants	*Anaeromyces* sp., (SR4) and *Neocallimastix* sp., (GR1) were used. 5ml (≈10^6 cfu/ml) of each fungus was used along with buffered inoculum to incubate wheat straw (500mg) for 48h and 72h respectively. Control i.e. substrates without fungi were provided.	The values ranged from the control to SR4; & GR1 incubated substrates respectively: • Increased IVDMD % (19.0 – 20.6; & **23.6**) after 48h; (25.0 – 30.7; & **34.4**) after 72h. • Inconsistent NDF digestibility % (13.3 – 10.9; & **13.7**) after 48h; (17.5-17.5; & **23.2**) after 72h. • Increased CMCase, xylanase, and cellobiase activity was recorded in all isolates while cellulose activity was only present in SR3, GR3 and GR1. GR1 recorded the highest cellulose and CMCase; SR4 recorded the highest xylanase; and SF1 recorded the highest cellobiase.
Shelke *et al.* (2009)	*In vitro* degradation of sugarcane bagasse based ruminant rations using anaerobic fungi	*Neocallimastix* sp., GR1 and *Piromyces* sp., WNG-12 were used. 5ml (≈2 x 10^3 zoospores/ml) of each fungus was used along with buffered inoculum to incubate sugarcane bagasse and TMR (Sugarcane bagasse + wheat straw + concentrate i.e.20:30:50) for 48h.	The values ranged from the control to GR1 & control to WNG-12 incubated substrates respectively: • Increased IVDMD % (22.48 – **27.98**; & 22.82 -26.51) for sugarcane bagasse; (**47.99 – 52.38**; & 49.61- 52.84) for TMR. • Increased NDF disappearance % (**17.62-28.05**; & 19.34-26.05) for bagasse; (**31.30-39.0**; & 33.85-37.44) for TMR.

		Control i.e. substrates without fungi were provided	• Increased ADF disappearance % (**23.44-31.18**; & 22.47-29.27) for bagasse; (**34.44-47.90**; & 32.07-42.29) for TMR. • Inconsistent TVFAs concentrations mmol/100ml (**8.91 – 10.57**; & 8.34-9.90) for bagasse; (**11.36-13.10**; & 10.50-12.14) for TMR.
Nagpal *et al.* (2011)	*In vitro* fibrolytic potential of anaerobic rumen fungi from ruminants and non-ruminant herbivores	*Orpinomyces* sp., RC1 & RB2; *Neocallimastix* sp., RG5; and *Caecomyces* sp., FE5 were used. 5ml ($\approx 10^6$ tfu/ml) of each fungus was used along with buffered inoculum to incubate wheat straw (500mg) for 48h and 72h respectively.	• Increased FPase, CMCase, cellobiase, and xylanase was recorded with FE5 recording the highest enzymes activity (21.4; 15.1, 37.4, & 26.0 mIU/ ml) respectively. • Increased IVDMD (%) at 48h (38.2 – **45.1 (FE5)**; 43.1 (RB2); 41.3 (RC1); 38.8(RG5)) and at 72h (38.6 – **48.9 (FE5);** 45.8 (RB2); 45.7 (RC1); 40.6 (RG5)). • Reduced NDF (%) at 48h (76 – **64.2 (FE5**); 66.1 (RB2); 68.5 (RC1); 72.7 (RG5)); and at 72h (74 – **61.3 (FE5)**; 62.9 (RB2); 64.4 (RC1); 70.8 (RG5)). ADF followed similar reduced pattern as NDF. • Increased TVFA (mmol/100ml) at 48h (8.3 – **12.7 (FE5**); 11.8 (RB2); 11.3 (RC1); 9.8 (RG5) and at 72h (8.5 – **13.6 (FE5)**; 12.4 (RB2); 12.0 (RC1); 10.8 (RG5).
Sirohi *et al.* (2013)	Isolation, characterization and fibre degradation potential of anaerobic rumen fungi from cattle	*Orpinomyces* sp., NFRI-1, NFRI-3, NFRI-7; NFRI-13; NFRI-17, NFRI-18, & NFRI-19. *Anaeromyces* sp., NFRI-2, NFRI-4, NFRI-5, NFRI-6, NFRI-8, NFRI-8; NFRI-9, NFRI-10, NFRI-11, NFRI-12, NFRI-14, NFRI-15, NFRI-15, NFRI-16, & NFRI-20. These fungi were isolated from cattle and 2ml of each isolate was used along with buffered inoculum to incubate wheat straw (375± 5 mg) for 48h	• Increased IVDMD in all isolates with **NFRI-17** recording the highest value (60.98%) that was similar to NFRI-3 (60.71%) and NFRI-13 (60.44%). • Increased partition factor (PF) with **NFRI-17** recording the highest factor (3.53) that was similar to NFRI-18 (3.48). • Increased microbial biomass yield (mg) with **NFRI-17** recording the highest value (82.79) that was similar to NFRI-18 (78.71). • Increased gas volume (ml) with **NFRI-14** recording the highest gas volume (71.33) similar to NFRI-5 (70.50). • Increased acetate concentration (mmol) in all isolates with **NFRI-17** recording the highest value (13.16) that was similar to NFRI-9 (12.83). • Insignificant propionate and butyrate concentrations (mmol) in isolates. • Increased CMCase, avicelase and xylanase activities was recorded in all isolates. Xylanase was highest in NFRI-1 (11.0 umol/m/h) at 96h; CMCase was highest in NFRI-18 (9.03umol/ml/h) at 96h; and

			avicelase was highest in all isolates at 120h with NFRI-4 and NFR1-7 recording the highest values (2.53 & 2.41umol/m/h) respectively.
Kumar *et al.* (2004)	*In vitro* degradation of cell wall and digestibility of cereal straws treated with anaerobic ruminal fungi	*Orpinomyces* sp., C-14, & B-13; *Piromyces* sp., C-15; & *Anaeromyces* sp., B-6 were used. 5ml of either 10^3 cfu/ml or 10^6 cfu/ml of each fungus was used along with buffer solution with or without strained rumen liquor (SRL) to incubate wheat straw and rice straw (500mg) respectively for 24h.	• Increased IVDMD by both fungal doses with 10^6 cfu/ml dose recording higher digestibility than 10^3 cfu/ml. • The use of SRL had no effect on IVDMD. • Increased IVDMD and reduced NDF, ADF and ADL (%) by all isolates compared to control. • The highest IVDMD in rice straw was recorded in B-6 (32.22 (con) – 45.24%); lowest NDF in B-13 (71.25 – 65.96%); lowest ADF (8.10 – 30.96%) and ADL (9.21 – 7.28 %) in C-14 respectively. • C-14 treated wheat straw recorded the highest IVDMD (38.48 – 52.73%), lowest NDF (79.34 – 69.71%), lowest ADF (40.17 – 29.01 %), and lowest ADL (12.32 – 7.96%).

1.3 Conclusion

Based on the above descriptions, it can be concluded that, despite variations in their extracellular enzymatic functions, both anaerobic and aerobic fungi are capable of increasing the utilization of low-quality substrates for better ruminant performance. Aerobic fungal pre-treatment of low-quality feeds can improve the antioxidant and anti-methanogenic potential of pre-treated forages and alter their metabolite composition. The literature to date has been focused on the use of aerobic fungi in pre-treating crop residues; information on aerobic fungal pre-treatment of grasses that stay long into the dry season is scarce. Even with respect to the use of crop residues, considerable variations in response to a particular fungus on the same substrate have been reported in the literature, indicating that fungal growth conditions apparently influence the performance of fungi on a substrate. Furthermore, there is only very limited information on the feasibility and effects of inoculating low-quality feeds such as silage with anaerobic fungi before introducing the ensiled forage into the rumen environment. Therefore, carrying out an investigation of pre-treating and ensiling low quality grasses along with some of the known crop residues with both aerobic and anaerobic fungi respectively may be a way forward towards improving low-quality forages for dry season feeding in tropical and sub-tropical countries.

1.4 Hypotheses

1. Various fungi have preferences in colonising forages or substrates with little nutritive value, especially those that are high in fibre or lignin contents, to grow and exhibit their fibrolytic and/or a lignolytic enzymatic functions.
2. Rumen fungal population is increased when highly fibrous and lignified feeds are fed.
3. The growth conditions used during the fungal pre-treatment of LQF are capable of affecting the activities of the aerobic fungi towards improving the LQF for dry season feeding.
4. Different fungal strains produce different effects on improving nutrient utilization of LQF. Both anaerobic and aerobic fungi can help in degrading lignocellulose contents of LQF. Aerobic fungi are likely to have more significant potential in degrading lignocellulose than anaerobic fungi, as they possess both ligninolytic and hydrolytic extracellular enzymatic functions.
5. The anaerobic and aerobic fungi are capable of improving chemical composition, digestibility and antioxidant potential of the pre-treated forages as well as reducing the secondary metabolite compositions of treated forages.

6. Anaerobic and aerobic fungal effects on low quality forages can affect rumen fermentation, resulting in increased nutrient degradability, total gas production, VFA profiles, ammonia content, as well as reduced methane gas production.

1.5 Study Objectives

1. To characterise the selected LQF for their chemical composition, secondary metabolites, antioxidant properties, *in vitro* degradability, fermentation profiles and total gas production to test the hypothesis if the forages are of a low nutritive quality that require fungal improvement.
2. To investigate the microbial composition of the *in vitro* degraded forages to test the hypothesis that forages with high structural components are capable of supporting microbial growth, especially fungi and their co-culture with bacteria.
3. To investigate some selected growth conditions that are capable of supporting the optimum growth and enzymatic function of selected white rot fungi (i.e. media, temperature, fermentation time) and isolated anaerobic fungi (media and fermentation time) to test the hypothesis that growth conditions have a great effect on the growth and enzymatic functions of the fungi towards improving the low-quality forages.
4. To investigate the ability of two selected white rot fungi (*Pleurotus ostreatus*; PO and *Ceriporiopsis rivulosus*; CR) in improving the nutritive value of selected mature forages (*A. gayanus*, *B. decumbens*, and *T. aestivum* straw) along with *L. perenne* that was used as a benchmark for the low-quality forages.
5. To investigate the ability of two anaerobic fungi to improve the nutritive value of selected matured forages (*A. gayanus*, *B. decumbens*, and *T. aestivum*) along with *L. perenne* that was used as a benchmark for the low-quality forages.
6. To investigate the fungal treated forages for their potential to modify *in vitro* degradability, fermentation profiles, methane and total gas production.

Chapter 2

Chemical Composition and *In Vitro* Degradability, Fermentation Profiles and Total Gas Production of Selected Forage Samples for Their Use in Ruminant Diets

2.1 Introduction

In most tropical and subtropical countries, during the prolonged dry season ruminant animal production and performance have been reduced due to unavailability of good quality green forages (Odenyo *et al.*, 1997; Van Saun, 2006). Presently during this dry season, energy and protein supplementation have been the option of most farmers, which includes the use of energy sources such as crop residues, agro-industrial by-products, perennial food crops, roots and tubers as well as protein sources in the form of fodder tree legumes. Some of these supplements are readily available as well as cheap sources of protein in the form of green foliage with high digestibility (Dzowela *et al.*, 1995; Fondevila *et al.*, 2002; Mota *et al.*, 2005; Franzel *et al.*, 2014). Moreover, these can contain secondary metabolites such as saponins and tannin ((Norton, 1994; Cudjoe and Mlambo, 2014; Franzel *et al.*, 2014) that are capable of reducing methane (i.e. a form of energy loss) production in ruminant animals (Bhatta *et al.*, 2013a). However, these supplements are either not capable of providing adequate nutrients to meet the energy (total volatile fatty acids) needs due to their high fibre, and lignin contents, and/or most of the protein (ammonia) is degraded by the rumen microbes leaving less by-pass protein for post-ruminal digestion, thus not meeting the nutrient requirement of high performing animals. On the other hand, the available green forages have not been adequately utilised as possible supplements to improve feed utilisation despite their immense benefits in maintaining better rumen function and providing vitamin A needed to maintain a balanced immune system and good health condition (Brum *et al.*, 2008; Elejalde *et al.*, 2010). There is, therefore, a need to search for possible ways of improving the available forages, with or without fodder tree legumes, to enhance ruminant production and performance during the dry season, using the temperate forages as a benchmark to place the results in perspective.

This current study has selected four out of the most available dry season green grasses, *Pennisetum purpureum* (PP), *Panicum maximum* (PM), *Brachiaria decumbens* (BD) and *Andropogon gayanus* (AG), and 2 woody legumes (*Leucaena Leucocephala* and *Gliricidia sepium*), as well as two British forages, i.e. a wheat straw (*Triticum aestivum*) and a grass (*Lolium perenne*). The selection of the Nigerian grasses were based on the fact that the grasses

have been found promising, while the legumes have been found to be used as supplements in animal feeds during the dry season. The British forages were selected and used as both positive and negative controls (i.e. forage controls) for the selected Nigerian grasses. The nutritive quality of the forages was assessed by their nutrient composition, *in vitro* rumen degradability and microbial composition. In this chapter, only the nutrient composition and *in vitro* rumen degradability were investigated while the ruminal microbial composition was examined in Chapter 3. The aim of the whole forage evaluation process was to ascertain the nutritive quality of the four Nigerian grasses and select two grasses that are most suitable for further biological upgrade. The selected grasses are to be used along with one legume that shows an higher potential to be used as supplementary feed. Also, to ascertain the nutritive quality of the British forages to confirm if they can be used as positive and negative forage controls in further studies.

The nutrient composition was determined primarily by chemical analyses, which involved proximate (Organic matter, OM; crude protein, CP; and ether extract, EE), detergent fibres (Acid detergent fibre, ADF; neutral detergent fibre, NDF; hemicellulose; cellulose; and acid detergent fibre, ADL) content and secondary metabolites (total phenols, TP; total tannins, TT; condensed tannin, CT; hydrolysable tannin, HT; and total antioxidant capacity, TAC) contents. The *in vitro* rumen organic matter degradability (IVOMD), total volatile fatty acids (TVFA), methane gas (CH_4), total gas production (tGP), and ammonia nitrogen (NH_3-N), were also measured (Menke and Steingass, 1988; Blümmel *et al.*, 1997) to assess the feeding value of these selected forages..

2.2 Objectives

To determine:

1. The chemical composition of the selected forage types using chemical analysis.
2. The nutrient degradability, fermentation parameters, total gas production of selected forages using *in vitro* ruminal fermentation technique.

2.3 Materials and Methods

2.3.1 *Sample collection and preparation*

Replicated (n=4) samples of four Nigerian grasses (PP, BD, AG and PM), 2 Forage controls (LP and TA straw) and two legumes (LL and GS) were used. The Nigerian forages were collected randomly from four different subplots of an experimental field at the Federal University of Agriculture, Abeokuta (FUNAAB), in March during the end of dry season. Each forage sample was cut at 2cm above the soil surface, bulked from each sub-plot, chopped, oven-dried at 55^{o}C and transported to Newcastle University. The samples were redried at 55^{0}C in a forced- air oven for 48 h and ground through a 1 mm sieve with the use of a grinder (Christy mill, Christy and Norris Ltd, Suffolk, United Kingdom) for further analyses. Appropriate amounts of Forage controls were obtained from another PhD researcher who was also studying at Newcastle University.

2.3.2 *Chemical analyses*

2.3.2.1 *Determination of Dry matter, Ether extract, Crude protein, Fibre fractions and Ash*

The various grass and legume samples were analysed in triplicate for Dry matter (DM), Ether extract (EE) using (AOAC, 1990) methods as described in Appendix 2.1**.** Nitrogen values were obtained using an Elementar vario macro cube (Elementar, Hanau Germany) analyser which involved using combustion, post-combustion and reduction tube in the furnace of the analyser. The analyser used about 0.1g of the sample in a thin foil cup, which was then carefully folded to remove any trapped air using a specialised tool provided for the elementar. The Crude protein (CP) content was then calculated (N x 6.25). Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Acid Detergent Lignin were assessed using the methods of Van Soest (1991) and Goering and Van Soest (1970) without using sodium sulphite and Dekalin as described in Appendix 2.2. The difference between NDFom and ADFom determined the concentration of hemicellulose, while the difference between ADFom and Lignin established the concentration of cellulose. Ash concentration was determined by complete combustion of the sample in a muffle furnace (Carbolite, CWF 1200 muffle furnace) at 550^{0}C for 5hrs (AOAC, 1990).

2.3.2.2 *Determination of total phenolics (TP) and total tannin (TT), Condensed tannin (CT), Hydrolysable tannin (HT)*

Total phenolic and total tannin contents were measured using modified Folin-ciocalteu method. This method involved the use of an insoluble matrix, polyvinyl polypyrrolidone (binds tannic-phenolics), that separates non-tannin phenols from tannin phenols, therefore making it easy to obtain tannin measurements. On the other hand, the butanol- HCl – iron method was used for analysing CT (Makkar, 2003). Both TP and TT were expressed as tannic acid equivalents, while CT was expressed as (-) - epigallocatechin equivalents. The calculated difference between the TT and CT was also shown as the Hydrolysable tannins (Singh *et al.*, 2005), see Appendix 2.3 for the detailed procedure.

2.3.2.3 *Determination of Antioxidants*

Antioxidant capacities of the samples were determined according to the Ferric Reducing Antioxidant Power assay (Benzie and Strain, 1996). Results obtained were then expressed as μmol Fe (II)/g dry weight of the sample.

2.3.3 *In vitro* incubation with the selected forage samples

Three sub experiments were conducted by using either 4 x 2 x 3 (Nigerian grasses) or 2 x 2 x 3 (Nigerian legumes) or 2 x 2 x 3 (forage controls) factorial arrangements, each with 2 replicates as well as the negative control. The experiments examined both the principal and factorial effects of four (4) grasses, two (2) legumes and two (2) forage controls respectively on rumen *in vitro* dry matter degradability (IVDMD), organic matter degradability (IVOMD), pH, NH_3N, CH_4, total gas production (tGP) and VFA profiles from rumen fluid, collected from 2 different sheep, during 3 different incubation times (12h, 48h and 72h). Negative controls (blank) contained the buffered rumen fluid with no substrate. The values from these blanks were used for the correction of gas produced from small particles and microbial residues present in the buffered ruminal fluid.

2.3.3.1 *Collection of rumen fluid*

Rumen fluid (RF) samples were obtained from four freshly slaughtered sheep at a slaughterhouse (Linden Foods LTD.) located at Burradon, Newcastle Upon Tyne, UK on the 31st of March, 2015. The sheep were reported to be fed grass silage-based total mixed ration (TMR) plus brassica based vegetable before slaughtering. Each of the two rumen fluid samples used was obtained by pooling together rumen fluid from two sheep fed the same diet (i.e. Sheep

1 & 4, and sheep 2 & 3). Immediately after slaughtering, the rumen was cut open, and RF was collected by squeezing the rumen contents through two layers of a muslin cloth, using a large funnel, into a pre-warmed insulated thermos flask. The thermos flasks were tightly closed to maintain anaerobic conditions inside the flasks. The flasks were transferred to the laboratory for immediate use within one hour of RF collection.

2.3.3.2 *Buffer solution*

This was prepared according to the synthetic saliva procedure of (McDougall, 1948). The chemicals in Table 5 were dissolved in a beaker containing distilled water on a hot magnetic stirring plate (at about 50^0C). The pH of the solution was between 8.5 -8.7, this was adjusted to pH values ranging between 7.0 – 7.2 by adding a few mls of 1M HCl (12mls / 5 litres). Before the start of the experiment, the solution was transferred into dark bottles, flushed with CO_2 to remove oxygen, screw-capped and kept in a water bath at 39^0C until mixed with RF as described in the following section.

Table 5 The chemical amounts used for different volumes of McDougall buffer solution

Chemicals	g/L distilled water	g/ 5L distilled water
$NaHCO_3$	9.8	49.0
$Na_2HPO_4.12H_2O$	9.3	46.5
NaCl	0.5	2.4
KCl	0.6	2.9
$CaCl_2$ anhydrous	0.0	0.2
$MgCl_2$ anhydrous	0.1	0.3

2.3.3.3 *Buffered inoculum*

Appropriate quantities of RF from each sheep were transferred quickly under two layers of muslin cloth into pre-warmed dark bottles (2.5 L capacity) containing buffer solution to achieve 1:2 ratio of RF: Buffer solution. The bottles containing the buffered RF were purged with CO_2 to remove oxygen and tightly closed with a dispenser (50 ml capacity, Fisher Scientific UK). The pH of each buffered inoculum was adjusted to around 7.0 ± 0.2. The bottles containing inoculum were kept in a water bath at 39^0C until used to incubate test samples. Part of the buffered inoculum without samples was taken and analysed for fermentation parameters

(i.e.ammonia, tVFAs and its molar concentrations). The buffer ammonia nitrogen content was 10.39 mg / L, tVFA concentration (35.89 mmol/L), acetate concentration (28.59 mmol / L), propionate concentration (3.93 mmol/L), butyrate concentration (1.50 mmol/L), isovaleric concentration (1.11 mmol/L), and valerate concentration (0.75 mmol/L). These analyses were carried to know the starting point of the fermentation and degradability process, although this should have been done with the investigated samples to be scientifically justified, to represent the 0h.

2.3.3.4 In vitro incubation, total gas production, fermentation parameters and microbial counts

About 0.2g of each of the ground forage samples was put into a 50 ml calibrated glass syringe fitted with a luer metal tip (MRS Scientific, SAMCO, UK). About 20 ml of the buffered inoculum was dispensed into it. Each forage sample was incubated in duplicate for each incubation time and RF from each sheep. This preparation was made for each of the three (3) incubation periods (i.e. 12, 48 and 72 hours) and the buffered RF from two different sheep. The piston that had already been slightly lubricated with vaseline was pushed upward, towards where the inoculum is, in the syringe for the complete elimination of air. At the other end of the syringe a four (4) way-male-slip stopcock (Cole Palmer Instrument, UK) was fitted to prevent the escape of the inoculum and the gas generated during the incubation period. The syringes were then placed in a water bath at 39^0C and were gently shaken every one hour during the first 8h of incubation and subsequently were swirled three (3) times in a day.

The tGP in each syringe was measured at 0, 3, 6, 9, 12, 15 18, 21, 24, 30, 36, 42, 48, 54, 60, 66 and 72hr. At the end of each selected incubation period (n=3), the fluid samples were drawn from each of the syringes into plastic bottles, the pH was determined immediately with the aid of a pH meter (Oakton Acorn series pH 6 metre, Sigma-Aldrich) and the syringes were transferred into an ice-box to stop fermentation.

The extent and rate of gas production, as well as the lag phase, were determined for each forage by fitting the gas production data obtained from each selected incubation period (n=3) to the non linear modified Gompertz equation (Schofield *et al.,* 1994; Lay *et al.,* 1998; Liu *et al.,* 2002; Patil *et al.,* 2012) using Matlab.

$$y = a \times \exp(-\exp((\cup * \exp(1)/a) * (L - x) + 1))$$

Where:

y= total volume of gas produced at any time t

a = maximum potential of gas yield (ml/ g OM)

U = rate of maximum gas production

L = length of lag phase (hours)

t = time at which total gas production y is calculated, hours

Samples for NH_3-N determination were prepared by pipetting 2ml of each fermented fluid sample into a capped-container (5ml centrifuge tube, Sigma Aldrich) and acidifying them with 2 ml of 1 N HCl (Bateman and Jenkins, 1998). This was then centrifuged at 2500 rpm for 10 minutes, and the supernatant was stored in a freezer (-20^0C).

Samples for methane and CO_2 gas determination were prepared by taking about 10 ml of the headspace gas in the 50 mL incubated syringes from one replicate/sample (as described further in 2.4.4).

A separate 2 ml sample of each fermented fluid was pipetted into a capped- container and acidified with 0.4 mL of metaphosphoric acid (0.25 w/v) as described by Erwin *et al.* (1961). This was centrifuged at 13000rpm for three (3) minutes, the supernatant was then collected and kept in a freezer (-20^0C) until ready for VFAs analyses.

Another batch of samples was also prepared and collected for microbial studies. About 0.4g of each of the ground forage samples was put into a 50 ml centrifuge tube. About 40 ml of the buffered inoculum was dispensed into it. Each forage sample was incubated in duplicate for 48h and this preparation was made for each of the buffered RF obtained from two different sheep. Further details are provided in Chapter 3.

2.4 Measurements

2.4.1 *In vitro degradability*

DM was measured by draining the remaining contents in the syringes into pre-weighed sintered crucibles attached to a vacuum suction for proper filtering; the syringes were rinsed with water to remove every residue. The residues in the sintered crucibles (porosity no 1) were dried at 105^0C overnight, while OM was measured by transferring the weighed dried residues in the

sintered crucibles into the muffle furnace for ashing at 550^0C for 6 hours. The calculation for IVDMD and IVOMD of each sample was determined by deducting the weight of DM and OM of residues from the initial DM and OM weights of the incubated samples (see Appendix 2.4 for further calculations

2.4.2 NH_3-N analysis

NH_3-N was analysed by an ABX Pentra 400 clinical analyser (Horriba Ltd, Kyoto, Japan) with calibrated standards of NH_3-N at 25, 50, and 100 µg / ml in pure distilled water. Sample dilution with pure distilled water was applied to keep unknown NH_3-N concentration within the range of the standards. The NH_3-N determination was based on a colorimetric method in which a blue-green colour was formed by the reaction of NH_3, sodium salicylate, sodium nitroprusside and sodium hypochlorite in a buffered alkaline solution, pH 12.8 – 13.0. The resulting colour due to NH_3-salycylate complexes was tested for absorbance at 660nm (Coudène *et al.*, 2005)

2.4.3 *VFA analysis*

2.4.3.1 *Chemicals*

In the QIC operation, 0.1M octanesulphonic acid (OSA) was purchased from DIONEX (Camberley, UK) and then diluted to give 1mM eluent. In obtaining the standard, 40% tetrabutylammonium hydroxide (TBAOH) was purchased from Sigma-Aldrich, UK and then diluted to 10 mM. Calibration of the instrument was done by using general purpose reagents. Certified standards for VFAs were purchased and run as unknown 10mg/l respectively. High purity water was used for all dilutions of the standards, samples and the eluents.

2.4.3.2 *Analytical procedures*

Before undergoing instrument analysis, potential column contaminants such as humic acids and aromatic carboxylic acids were removed from the samples by passing the samples through a Dionex On-Guard-P cartridge**.** Determination of some VFAs, such as iso-valerate and iso-butyrate which were usually quantified, required that interferences caused by carbonate must be avoided and valerate, hexanoate and heptanoate required the removal of chloride for their peaks to be observed. This was done by using octanesulphonic acid and through sonication as described below for the VFA's sample preparation.

2.4.3.3 *Sample preparation*

The samples prepared for VFAs analyses were investigated using an ion exchange chromatograph based on a modified method of Manning and Bewsher (1997). Aqueous samples were syringe-filtered through 0.2 µm filters, acidified 1:1 v/v with oxysulfonic acid and

sonicated in a sonic bath for 30 minutes to remove carbonate from the samples as carbon dioxide. The resulting samples were analysed on a DIONEX ICS-1000 equipped with an Ionpac ICE-AS1, 4 × 250 mm column using a 1.0 mM heptafluorobutyric acid eluent solution. The volume of the injection loop was 10 µl, and the flow rate was 0.16 ml/min; see details for calculations in Appendix 2.5.

2.4.4 *CH_4 determination*

The gas in the syringes was immediately transferred into a 12 mL evacuated container (Exetainer, Labco Limited, High Wycombe, Buckinghamshire, UK) by passing an injection needle through one of the openings of the 4-way male-slip stopcock, and this then was analysed for methane concentration. Methane was monitored in the gas phase as % by volume, using gas chromatography (GC) (Carlo Erba HRGC S160 GC). Samples of gas (100 µl) were removed from the incubated gas produced, that was captured in the evacuated containers using gastight syringes (SGE-Europe Ltd., Milton Keynes, UK) and injected directly onto the GC fitted with an FID detector and HP-PLOTQ column (0.32 mm diameter, 30 m length and 20 µm film; Agilent, Wokingham, UK). Hydrogen was used as the carrier gas at a flow rate of 250 ml/min and the oven temperature was 35^0C.

2.5 Statistical Analysis

One way ANOVA was used to analyse the chemical composition of each forage type (i.e. Nigerian grasses, legumes and forage controls). All data sets for the *in vitro* study were statistically analysed by following the factorial designs according to the General Linear Model procedure on Minitab 16 software to determine both the main and interactive effects of each forage type (i.e. Nigerian grasses, Nigerian legumes and Forage controls) and sheep RFs separately on the IVDMD, IVOMD, NH_3-N, VFA's profiles, CH_4, pH and tGP at each incubation time (12h, 48h, and 72 hours). A $P < 0.05$ was considered to indicate statistical significance for different effects. Various means were separated using Tukeys *post-hoc* test and the differences between means were declared significant if $P < 0.05$. Relationships between chemical composition, secondary metabolites, gas production, gas production constants, in vitro organic matter degradability, ammonia concentration, methane concentration and volatile fatty acids were analysed using pearson correlation of Minitab 16 software.

2.6 Results

2.6.1 *The chemical compositions of the selected forages*

The chemical compositions of the various forage types are shown in Table 6. The chemical content (g/ kg OM) of each forage type significantly ($P < 0.05$) varied.The Nigerian grasses were low in CP (i.e. < 80 g/ kg OM), with an exception in AG, and EE (i.e. < 20 g/ kg 0M); high in fibre (i.e. NDF > 700g / kg OM and ADF > 500 g/ kg OM) and lignin (i.e. > 80 g/ /kg OM); and low in secondary metabolites with the highest content recorded in AG than other grasses. The legumes were very high in CP (i.e. > 240 g/kg OM) and EE (i.e. > 30 g / kg OM); low in fibre contents (i.e. NDF < 500g / kg OM and ADF < 350 g/ kg OM); high in lignin (i.e. > 80 g/ /kg OM); and high in secondary metabolites, with higher content recorded in LL than GS. The forage controls varied drastically in their chemical contents. LP recorded higher CP and EE, and had lower fibre and lignin contents than TA, while the opposite was recorded in TAS. Their secondary metabolites content was similar, with an exception in TP and TAC where LP recorded higher values than TA.

Table 6 Mean chemical compositions (g / kg OM) of the selected forages alongside standard error of means (SEM) for each group of forages

Composition	**Grasses**					**Legumes**			**Forage controls**			
	PP	PM	BD	AG	SEM	GS	LL	SEM	LP	TA	SEM	Pooled SEM
Organic matter	865.97^{d}	885.66^{c}	924.22^{a}	915.47^{b}	7.04	883.78^{b}	908.22^{a}	5.48	907.92^{b}	942.27^{a}	7.7	4.8
Proximate and Fibre fractions (g / kg OM)												
Crude protein	75.78^{b}	69.39^{c}	63.79^{c}	128.12^{a}	7.9	243.27^{b}	280.77^{a}	9.2	130.79^{a}	29.85^{b}	22.6	17.6
Ether extract	14.98^{a}	10.36^{b}	11.83^{b}	16.59^{a}	0.8	31.14^{b}	63.62^{a}	7.3	19.21^{a}	11.57^{b}	1.7	3.5
NDF	800.84^{a}	787.98^{a}	797.32^{a}	749.47^{b}	6.4	492.99^{a}	384.23^{b}	24.5	500.98^{b}	825.90^{a}	72.7	34.6
ADF	569.27^{a}	582.34^{a}	577.96^{a}	570.03^{a}	4.2	316.25^{a}	227.35^{b}	19.9	392.33^{b}	588.19^{a}	44.6	28.3
ADL	83.30^{c}	145.14^{a}	82.72^{c}	103.98^{b}	8.0	192.60^{a}	110.23^{b}	18.5	48.44^{b}	156.81^{a}	24.3	9.2
Hemicellulose	231.57^{a}	205.64^{ab}	219.37^{a}	179.44^{b}	7.6	176.75^{a}	156.88^{a}	6.2	108.65^{b}	237.71^{a}	29.6	9.0
Cellulose	485.97^{a}	437.21^{b}	495.24^{a}	466.05^{ab}	7.8	123.65^{a}	117.13^{a}	2.0	343.88^{b}	431.38^{a}	21.4	30.7
Secondary metabolites (g / kg OM)												
Total phenols (TP)	10.90^{b}	7.41^{d}	9.03^{c}	17.86^{a}	1.2	30.66^{b}	119.57^{a}	19.9	11.78^{b}	5.09^{a}	2.1	7.5
Total tannins (TT)	6.91^{b}	2.97^{c}	3.29^{c}	14.33^{a}	1.4	21.76^{b}	95.10^{a}	16.4	4.04^{a}	2.37^{a}	0.7	6.2
Condensed tannins (CT)	5.20^{b}	1.99^{c}	1.91^{c}	9.40^{a}	1.0	20.97^{b}	67.38^{a}	11.0	2.73^{a}	1.51^{a}	0.4	4.5
Hydrolysable tannins (HT)	1.72^{b}	0.98^{b}	1.38^{b}	4.93^{a}	0.5	0.79^{b}	27.72^{a}	6.7	1.30^{a}	0.86^{a}	0.4	2.0
Total antioxidant capacity (TAC)	4.28^{c}	4.41^{c}	5.10^{b}	7.40^{a}	0.4	3.24^{b}	42.24^{a}	8.7	6.30^{a}	4.11^{b}	0.5	2.6

Means with different letters in the same row were significantly different ($P < 0.05$) for each forage group; PP (*P. purpureum*), PM (*P. maximum*), BD (*B. decumbens*), AG (*A. gayanus*), GS (*G. sepium*), LL (*L. leucocephala*), LP (*L. perenne*), TA (*T. aestivum*), NDF (neutral detergent fibre), ADF (Acid detergent fibre), ADL (Acid detergent lignin), SEM (Standard error of means).

2.6.2 *Degradability, fermentation profiles and total gas production of the selected forages*

2.6.2.1 *IVDMD and IVOMD*

The interactive effects of each forage type and sheep RF on the IVDMD (g/kg DM) and IVOMD (g/kg DM) at 12h, 48h, and 72h of incubation are presented in Table 7 and 8. The interaction presented significant ($P < 0.05$) effects on the IVDMD and IVOMD values. The IVDMD and IVOMD values obtained from the interactions increased as the incubation time increased. Most forages in each group recorded IVDMD and IVOMD (g / kg DM) less than 550 g/ kg after 72h of incubation with a few exceptions in GS (i.e. > 550 g / kg) and LP (i.e. > 650 g / kg). The nutrient degradability of each forage within each forage type followed this order after 72h of incubation: for the Nigerian grasses (AG and PP > BD and PM); for the legumes (GS > LL); and for the forage controls (LP > TA). Also, the Nigerian grasses and TA recorded an higher increase in nutrient degradability from 12h to 48h than from 48h to 72h of incubation.The legumes and LP recorded higher nutrient degradability at 12h of incubation than the Nigerian grasses and TA. The sheep RF used for incubating each forage type had no significant effect on the IVDMD and IVOMD values at various selected incubation hours.

Table 7 The interactive effects of forage type and sheep RF on the mean IVDMD (g/kg DM) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	278.10	450.79^{ab}	517.90^{a}
P. maximum	270.66	598.80^{c}	426.28^{b}
B. decumbens	269.83	421.55^{bc}	456.15^{b}
A. gayanus	278.52	473.21^{a}	524.62^{a}
Sheep B			
P. purpureum	282.15	453.67^{ab}	521.80^{a}
P. maximum	277.38	419.61^{bc}	447.07^{b}
B. decumbens	276.46	435.47^{abc}	468.87^{b}
A. gayanus	283.20	478.45^{a}	530.54^{a}
SEM	26.6	19.9	28.9
	Legumes		
Sheep A			
G. sepium	447.73	591.86^{a}	596.91^{b}
L. leucocephala	405.78	419.09^{b}	442.47^{d}
Sheep B			
G. sepium	465.38	618.03^{a}	626.68^{a}
L. leucocephala	422.58	432.03^{b}	455.83^{c}
SEM	21.1	68.6	62.1
	Forage control		
Sheep A			
L. perenne	471.95^{a}	684.92^{a}	691.08^{a}
T. aestivum	107.84^{b}	345.34^{b}	388.50^{b}
Sheep B			
L. perenne	477.46^{a}	692.09^{a}	701.14^{a}
T. aestivum	136.53^{b}	354.07^{b}	396.67^{b}
SEM	133.8	128.2	115.4
Pooled SEM	82.1	75.5	68.0

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean.

Table 8 The interactive effects of forage type and sheep RF on the IVOMD (g/kg DM) at each incubation time (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	281.90	480.38	526.84[a]
P. maximum	277.84	435.75	442.25[b]
B. decumbens	280.34	438.63	468.49[b]
A. gayanus	281.85	495.42	530.50[a]
Sheep B			
P. purpureum	285.38	481.47	530.53[a]
P. maximum	282.61	448.42	458.32[b]
B. decumbens	284.96	457.94	482.85[b]
A. gayanus	289.98	499.10	536.83[a]
SEM	27.4	19.3	26.7
	Legumes		
Sheep A			
G. sepium	456.62	605.23[b]	623.33[b]
L. leucocephala	413.06	460.33[c]	468.04[c]
Sheep B			
G. sepium	474.63	638.65[a]	653.91[a]
L. leucocephala	430.04	463.47[c]	468.83[c]
SEM	21.9	61.3	66.9
	Forage control		
Sheep A			
L. perenne	482.62[a]	691.81[a]	697.53[a]
T. aestivum	148.08[b]	361.21[b]	418.29[b]
Sheep B			
L. perenne	486.25[a]	710.43[a]	718.14[a]
T. aestivum	168.85[b]	375.43[b]	421.97[b]
SEM	123.6	126.0	109.5
Pooled SEM	78.5	72.6	67.1

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean

2.6.2.2 *NH_3-N concentrations*

The interactive effects of each forage type and sheep RF on the NH_3-N concentrations (mg/L) at 12h, 48h, and 72h of incubations are presented in Table 9. The interaction between each forage type and sheep RF had significant ($P < 0.05$) effects on the NH_3-N concentrations. Among each forage type, the highest NH_3-N contents (76.97, 90.81 and 87.19 mg / l) were recorded in AG, GS and LP respectively, while the lowest NH_3-N contents (30.43, 40.57, and 16.20 mg / l) were recorded in PM, LL and TA respectively. Most forages in each group recorded NH_3-N content less than 50 mg / l, with the exception of those forages that recorded the highest NH_3-N contents from 48h – 72h of incubation. Among those forages that recorded the highest NH_3-N content above 50 mg / l, the Nigerian forages (i.e. AG and GS) released NH_3-N earlier between 0 - 12h than the UK grass (LP).

Table 9 The interactive effects of forage type and sheep RF on the NH_3-N concentrations (mg/l) at different incubation times (12, 48 and 72 hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	20.15[b]	25.11[b]	38.25[b]
P. maximum	18.77[b]	21.07[b]	30.43[b]
B. decumbens	22.15[b]	25.67[b]	32.04[b]
A. gayanus	55.99[a]	64.97[a]	74.92[a]
Sheep B			
P. purpureum	22.37[b]	28.24[b]	40.60[b]
P. maximum	21.26[b]	24.11[b]	33.51[b]
B. decumbens	23.73[b]	26.39[b]	35.93[b]
A. gayanus	57.32[a]	71.90[a]	76.97[a]
SEM	11.6	14.1	13.2
	Legumes		
Sheep A			
G. sepium	59.15[a]	78.19[a]	89.72[a]
L. leucocephala	15.31[b]	29.91[b]	40.57[b]
Sheep B			
G. sepium	61.66[a]	80.46[a]	90.81[a]
L. leucocephala	16.35[b]	35.86[b]	47.90[b]
SEM	17.0	17.9	17.6
	Forage control		
Sheep A			
L. perenne	32.97[a]	75.12[a]	86.31[a]
T. aestivum	7.08[b]	11.15[b]	16.20[b]
Sheep B			
L. perenne	40.59[a]	75.27[a]	87.19[a]
T. aestivum	7.10[b]	12.90[b]	17.87[b]
SEM	11.5	23.9	26.4
Pooled SEM	13.3	18.2	18.8

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean; NH_3-N, ammonia nitrogen.

2.6.3 *VFA profiles*

2.6.3.1 *Total VFA (tVFA)*

The interaction of forage type and sheep RF had a significant ($P < 0.05$) effect on the tVFA concentrations (mmol / L) at the various incubation hours (i.e. 12h, 48h, and 72h), as presented in Table 10.The tVFA in each forage type increased with increase in incubation time. On average, the tVFA production was 69.16, 155.94, and 179.36 mmol / L between 0-12h, 12-48h and 48-72h respectively, with the highest content from UK grass (LP), and the lowest from LL (legume) after 72h of incubation. It was observed that the legume that recorded the least tVFA contained the highest secondary metabolites. Initially, the legumes recorded higher tVFA content (0 -12h) than other forage types, but the differences between each forage type diminished after 12h. Among each forage type, the highest tVFA content was recorded in AG (Nigerian grass; 207.43 mmol / L), GS (Legume; 204.44 mmol / L), and LP (UK forage; 227.70 mmol / L) and the lowest tVFA content was recorded in PM (Nigerian grass; 158.06 mmol / L), LL (Legume; 136.07 mmol / L), and TA (UK forage; 151.83 mmol / L). The effect that the sheep RFs used in incubating had on tVFA concentration (mmol / L) was similar at each selected incubation time.

Table 10 The interactive effects of forage type and sheep RF on the total volatile fatty acids (tVFA concentration; mmol/L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	45.29^{b}	149.27^{ab}	175.48
P. maximum	43.69^{b}	117.11^{b}	158.06
B. decumbens	49.78^{ab}	128.50^{b}	170.12
A. gayanus	52.59^{ab}	183.44^{a}	188.22
Sheep B			
P. purpureum	58.85^{ab}	151.40^{ab}	195.83
P. maximum	53.40^{ab}	125.90^{b}	168.32
B. decumbens	60.19^{ab}	135.92^{b}	185.56
A. gayanus	67.12^{a}	188.82^{a}	207.43
SEM	0.9	3.8	3.1
	Legumes		
Sheep A			
G. sepium	83.91^{b}	163.09^{ab}	184.53^{ab}
L. leucocephala	57.13^{c}	122.29^{c}	136.07^{c}
Sheep B			
G. sepium	99.86^{a}	193.47^{a}	204.44^{a}
L. leucocephala	79.87^{b}	137.36^{bc}	145.82^{bc}
SEM	2.1	4.2	4.3
	Forage control		
Sheep A			
L. perenne	107.17^{a}	203.19^{a}	208.11^{a}
T. aestivum	28.56^{b}	115.97^{b}	151.83^{b}
Sheep B			
L. perenne	118.43^{a}	215.84^{a}	227.70^{a}
T. aestivum	39.51^{b}	130.07^{b}	169.30^{b}
SEM	6.1	6.8	4.5
Pooled SEM	3.5	4.8	3.8

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean.

2.6.3.2 *Acetate*

The interaction of forage type and sheep RF had a significant ($P < 0.05$) effect on the acetate concentrations (mmol / L) at 12h, 48h, and 72h of incubation (Table 11). The acetate concentration of each forage type increased with increase in incubation time, with the highest concentration (mmol / L) found in a legume (GS; 162.26) and straw (TA; 134.68); followed by LL and LP; and lastly by the Nigerian grasses (AG, PP, BD and PM) after 72h of incubation. The Nigerian grasses' acetate concentration was different at 12h, after which the differences diminished. Among the legumes, GS recorded higher concentration than LL at 12h, 48h, and 72h of incubation respectively. Among the forage controls, LP recorded higher acetate concentration at 12h and 48h of incubation than TA, after which TA recorded higher concentration (134.68 mmol / L) than LP after 72h of incubation. The effect of sheep rumen fluids on acetate concentration (mmol / L) were similar at each selected incubation time with sheep B rumen fluid recording the highest acetate concentration at 72h of incubation.

Table 11 The interactive effects of forage type and sheep RF on acetate concentration (mmol / L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	37.38^{b}	88.11	95.58
P. maximum	35.79^{b}	70.47	90.58
B. decumbens	39.73^{ab}	74.99	95.39
A. gayanus	42.19^{ab}	103.96	103.33
Sheep B			
P. purpureum	47.77^{ab}	89.08	107.29
P. maximum	43.53^{ab}	73.21	97.23
B. decumbens	49.73^{ab}	75.77	102.73
A. gayanus	55.16^{a}	104.31	115.90
SEM	0.8	2.1	1.6
	Legumes		
Sheep A			
G. sepium	70.80^{b}	130.34^{ab}	147.97^{ab}
L. leucocephala	47.68^{c}	101.64^{c}	114.83^{b}
Sheep B			
G. sepium	83.05^{a}	155.29^{a}	162.27^{a}
L. leucocephala	66.58^{c}	113.65^{bc}	121.08^{b}
SEM	1.7	3.1	3.0
	Forage control		
Sheep A			
L. perenne	63.33^{a}	108.24	109.03^{b}
T. aestivum	23.77^{b}	97.37	121.60^{ab}
Sheep B			
L. perenne	71.27^{a}	113.78	119.98^{ab}
T. aestivum	33.05^{b}	107.98	134.68^{a}
SEM	3.0	1.4	1.2
Pooled SEM	2.2	3.3	2.9

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean

2.6.3.3 *Propionate*

The interaction of forage type and sheep RF had a significant ($P<0.05$) effect on the propionate concentration (mmol/L) at 12h, 48h, and 72h of incubation (Table 12). The propionate concentration (mmol/L) increased as the incubation time increased. The highest propionate concentration (mmol/L) was recorded in the grasses. The UK grass (LP) gave higher concentration (86.00) than the Nigerian grasses. The lowest propionate concentration, that ranged between 15.02-28.99 mmol/L, was recorded in the legumes (GS and LL) and the straw (TA) after 72h of incubation. The Nigerian grasses were different in propionate concentrations at 12h and 48h, but the differences diminished at 72h of incubation. The UK grass produced higher propionate concentration at an earlier incubation time (12h) up to 48h than Nigerian grasses, that only showed a rapid increase in concentration from 48h of incubation. The legumes showed higher propionate concentration at 12h than Nigerian grasses and straw, but did not produce more at 48h and 72h of incubation respectively. The effect of sheep RF on propionate concentration (mmol/L) was similar at each selected incubation time, with sheep A RF recording the highest propionate concentration.

Table 12 The interactive effects of forage type and sheep RF on propionate concentration (mmol / L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	5.37[b]	52.49[b]	71.29
P. maximum	4.87[b]	42.91[b]	61.60
B. decumbens	5.29[b]	47.77[b]	67.02
A. gayanus	5.61[b]	69.62[a]	74.67
Sheep B			
P. purpureum	7.08[a]	52.56[b]	78.30
P. maximum	5.14[b]	46.46[b]	63.50
B. decumbens	5.50[b]	49.15[b]	70.72
A. gayanus	7.15[a]	72.69[a]	78.99
SEM	0.1	1.6	1.4
	Legumes		
Sheep A			
G. sepium	7.68[b]	22.65[b]	24.90[a]
L. leucocephala	5.54[c]	14.52[c]	15.02[b]
Sheep B			
G. sepium	10.25[a]	26.94[a]	28.99[a]
L. leucocephala	8.05[b]	17.18[c]	16.63[b]
SEM	0.3	0.7	0.9
	Forage control		
Sheep A			
L. perenne	38.43[a]	77.28[a]	80.51[a]
T. aestivum	2.62[b]	15.43[b]	22.14[b]
Sheep B			
L. perenne	40.14[a]	82.63[a]	86.00[a]
T. aestivum	3.10[b]	17.91[b]	25.15[b]
SEM	2.8	4.8	4.5
Pooled SEM	1.6	3.3	3.8

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean.

2.6.3.4 *Butyrate*

The interaction of forage type and sheep RF had a significant (P<0.05) effect on the butyrate concentrations (mmol/L) at 12h, 48h, and 72h of incubation (Table 13). The butyrate concentration (mmol/L) of each forage type increased slightly as the incubation time increased. In general, the highest and the lowest butyrate concentrations were recorded in LP (13.34) and LL (3.52) respectively at 72h of incubation. Among the Nigerian grasses, legumes, and forage controls, the highest concentrations were recorded in AG, GS, and LP respectively. However, the higher value (9.18) recorded in AG was comparable to that of BD (9.07). The effect of sheep RFs on butyrate concentration (mmol/L) was similar at each selected incubation time, with sheep B rumen fluid recording the highest butyrate concentration.

Table 13 The interactive effects of forage types and sheep RFs on butyrate concentration (mmol/L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	1.15^{b}	6.77^{ab}	6.67^{cd}
P. maximum	1.07^{b}	1.87^{c}	4.08^{e}
B. decumbens	1.20^{b}	3.60^{c}	4.86^{de}
A. gayanus	1.24^{b}	6.79^{ab}	7.05^{bc}
Sheep B			
P. purpureum	2.01^{a}	8.11^{a}	8.69^{ab}
P. maximum	1.71^{ab}	4.29^{bc}	5.56^{cde}
B. decumbens	2.03^{a}	8.61^{a}	9.07^{a}
A. gayanus	2.07^{a}	8.74^{a}	9.18^{a}
SEM	0.03	0.4	0.2
	Legumes		
Sheep A			
G. sepium	2.25^{b}	6.20^{a}	7.48^{b}
L. leucocephala	1.54^{c}	3.33^{b}	3.52^{d}
Sheep B			
G. sepium	2.92^{a}	6.70^{a}	8.37^{a}
L. leucocephala	2.34^{b}	3.39^{b}	4.89^{c}
SEM	0.1	0.2	0.3
	Forage control		
Sheep A			
L. perenne	3.14^{ab}	10.01^{b}	10.42^{ab}
T. aestivum	0.76^{b}	2.19^{c}	6.71^{b}
Sheep B			
L. perenne	4.39^{a}	11.59^{a}	13.34^{a}
T. aestivum	1.70^{ab}	3.14^{c}	8.35^{ab}
SEM	0.2	0.6	0.4
Pooled SEM	0.1	0.4	0.3

Means with different letters in the same column are significantly (P < 0.05) different; SEM, standard error of mean

2.6.3.5 *Isovaleric*

The interaction of forage type and sheep RF had a significant (P<0.05) effect on the isovaleric concentrations (mmol/L) at 12h, 48h, and 72h of incubation (Table 14). In general, the isovaleric concentration increased slightly from 12h-48h of incubation and the highest isovaleric concentration (mmol/L) was recorded in the UK grass (LP;2.05) and legume (GS; 1.95), followed by LL (1.05) and AG (1.00), other Nigerian grasses, and lastly by TA (0.00) after 72h of incubation. Among the Nigerian grasses, legumes and forage controls, the highest isovaleric concentration was recorded in AG, GS, and LP respectively.The results showed that forages with higher CP contents recorded higher isovaleric concentration. The sheep RFs used in incubating the forage types had no significant effect on the isovaleric concentration (mmol/L).

Table 14 Interactive effects of forage type and sheep RF on isovaleric concentration (mmol/L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	0.95^{b}	0.27^{bc}	0.59^{ab}
P. maximum	0.97^{b}	0.64^{ab}	0.72^{a}
B. decumbens	1.09^{ab}	0.65^{ab}	0.67^{ab}
A. gayanus	1.01^{ab}	0.93^{a}	0.94^{a}
Sheep B			
P. purpureum	1.21^{ab}	0.00^{c}	0.00^{b}
P. maximum	1.21^{ab}	0.76^{ab}	0.80^{a}
B. decumbens	1.30^{a}	0.67^{ab}	0.73^{a}
A. gayanus	1.22^{ab}	0.93^{a}	1.00^{a}
SEM	0.01	0.03	0.03
	Legumes		
Sheep A			
G. sepium	1.15^{b}	1.41^{b}	1.51^{b}
L. leucocephala	0.79^{c}	0.70^{c}	0.73^{d}
Sheep B			
G. sepium	1.41^{a}	1.84^{a}	1.95^{a}
L. leucocephala	1.06^{b}	1.00^{c}	1.05^{c}
SEM	0.03	0.1	0.1
	Forage control		
Sheep A			
L. perenne	0.93^{b}	1.96^{a}	1.96^{b}
T. aestivum	0.88^{b}	0.45^{b}	0.51^{c}
Sheep B			
L. perenne	1.15^{a}	2.03^{a}	2.05^{a}
T. aestivum	1.13^{a}	0.50^{b}	0.00^{d}
SEM	0.01	0.1	0.1
Pooled SEM	0.02	0.1	0.1

Means with different letters in the same column are significantly (P < 0.05) different; SEM, standard error of mean

2.6.3.6 *Valeric*

The interaction of forage type and sheep RF had a significant (P<0.05) effect on the valeric concentrations (mmol / L) at 12h, 48h, and 72h of incubation (Table 15). The valeric concentration increased with increase in incubation time. In general, the highest valeric concentration was recorded in the UK grass (LP), followed by legumes (GS > LL), then the Nigerian grasses (AG and BD > PP > PM), and lastly by the UK straw (TA) after 72 h of incubation. Among the Nigerian grasses, legumes and forage controls, the highest valeric concentrations (mmol / L) were recorded in AG (2.36) and BD (2.31); GS (2.87) and LP (6.33) respectively. However, the valeric concentration was not significantly affected by the sheep rumen fluids used in incubating the forage types.

Table 15 Interactive effects of forage type and sheep RF on valeric acid concentration (mmol / L) at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	12h	48h	72h
	Grasses		
Sheep A			
P. purpureum	0.44^{c}	1.55	1.35^{bc}
P. maximum	0.80^{bc}	1.22	1.08^{c}
B. decumbens	1.37^{a}	1.49	2.19^{ab}
A. gayanus	1.49^{a}	2.14	2.24^{a}
Sheep B			
P. purpureum	0.77^{bc}	1.66	1.55^{abc}
P. maximum	0.97^{b}	1.18	1.22^{c}
B. decumbens	1.42^{a}	1.72	2.31^{a}
A. gayanus	1.53^{a}	2.16	2.36^{a}
SEM	0.1	0.1	0.1
	Legumes		
Sheep A			
G. sepium	2.02^{ab}	2.50	2.67
L. leucocephala	1.59^{b}	2.11	1.98
Sheep B			
G. sepium	2.23^{a}	2.70	2.87
L. leucocephala	1.84^{ab}	2.15	2.18
SEM	0.04	0.04	0.1
	Forage control		
Sheep A			
L. perenne	1.35^{a}	5.69^{a}	6.20^{a}
T. aestivum	0.18^{b}	0.53^{b}	0.88^{b}
Sheep B			
L. perenne	1.49^{a}	5.80^{a}	6.33^{a}
T. aestivum	0.21^{b}	0.55^{b}	1.12^{b}
SEM	0.1	0.4	0.4
Pooled SEM	0.1	0.2	0.2

Means with different letters in the same column are significantly (P < 0.05) different; SEM, standard error of mean

2.6.4 *pH*

Table 16 shows the interactive effect of forage type and sheep RF on the pH values at 12h, 48h, and 72h of incubation. The interaction between the forage type and sheep RF produced a similar effect (P > 0.05) on the pH value of the Nigerian grasses and legumes but had a significant impact on the pH value of the forage controls after 72h of incubation. The effect of the sheep RF used in incubating the various forage types on the pH was insignificant (P > 0.05). The pH value decreased as the incubation time increased. On average, the pH values were 6.94, 6.80, and 6.76 between 0 – 12h, 12h - 48h, and 48h – 72h respectively. The highest pH was from the legumes and PM, and the lowest pH was from LP and AG. The Nigerian grasses and UK straw (TA) recorded more significant reduction in pH up till the 72h of incubation, but the legumes and the UK grass (LP) recorded a slower reduction in pH after 48h of incubation. The Nigerian grass (BD) and UK grass (LP) recorded an earlier decline in pH (0 -12h) than other grasses.

Table 16 Interactive effects of forage type and sheep RF on the pH values at different incubation times (12, 48 and 72hours)

Interactive effect of forage types and sheep rumen fluid	**12h**	**48h**	**72h**
	Grasses		
Sheep A			
P. purpureum	7.01	6.76^{bc}	6.70
P. maximum	7.01	6.90^{a}	6.83
B. decumbens	6.95	6.80^{abc}	6.77
A. gayanus	7.01	6.76^{bc}	6.66
Sheep B			
P. purpureum	6.98	6.76^{bc}	6.69
P. maximum	6.99	6.86^{ab}	6.81
B. decumbens	6.93	6.77^{abc}	6.74
A. gayanus	6.98	6.71^{c}	6.66
SEM	0.03	0.1	0.1
	Legumes		
Sheep A			
G. sepium	6.98	6.88	6.84
L. leucocephala	7.05	6.88	6.85
Sheep B			
G. sepium	6.93	6.87	6.80
L. leucocephala	6.94	6.86	6.84
SEM	0.1	0.04	0.03
	Forage control		
Sheep A			
L. perenne	6.74^{b}	6.65b	6.65^{b}
T. aestivum	7.01^{a}	6.88a	6.78^{a}
Sheep B			
L. perenne	6.73^{b}	6.64b	6.64^{b}
T. aestivum	7.00^{a}	6.83a	6.75^{a}
SEM	0.1	0.1	0.1
Pooled SEM	0.1	0.1	0.1

Means with different letters in the same column are significantly (P < 0.05) different; SEM, standard error of means

2.6.5 *Methane (CH_4) concentration*

Table 17 presents the interactive effects of forage type and sheep RF on methane concentration, as expressed in L/kg DDM (digested dry matter) and L/kg DOM (digested organic matter), at different incubation times. The interaction of the forage type and sheep RF had a significant effect on the methane concentration (L/kg DDM and L/kg DOM) of Nigerian grasses and legumes, but produced a similar effect on the forage controls after 72h of incubation. The sheep RF had no significant effect on the methane concentration (L/kg DDM and L/kg DOM). The methane concentration (L/kg DDM and L/kg DOM) increased as the incubation time increased. In general, the methane content of the forage types after 72h of incubation increased based on this order: for the Nigerian grasses (AG > PP > BD > PM), for legumes (GS > LL), and for the forage controls i.e. TA and LP, they produced similar methane content. The legume that recorded the least methane content also had the highest secondary metabolites content.

Table 17 Interactive effects of forage type and sheep RF on methane (CH_4) concentration (ml/g digested DM & ml/g digested OM) at different incubation times (12, 48 and 72h)

Forage types	12h (ml/g DDM)	48h (ml/g DDM)	72h (ml/g DDM)	12h (ml/g DOM)	48h (ml/g DOM)	72h (ml/ g DOM)
Interactive effect of sheep RF and forage type						
			Grasses			
Sheep A						
PP	1.02^{b}	38.02^{b}	49.64^{bc}	1.13^{b}	40.28^{a}	57.84^{ab}
PM	0.84^{b}	28.50^{c}	35.62^{e}	0.84^{b}	26.69^{b}	38.59^{d}
BD	0.90^{b}	31.47^{c}	46.09^{cd}	0.89^{b}	31.16^{b}	48.56^{bcd}
AG	1.10^{b}	41.48^{ab}	58.46^{ab}	1.13^{b}	41.14^{a}	63.15^{a}
Sheep B						
PP	1.37^{ab}	38.65^{b}	53.96^{abc}	1.54^{ab}	41.03^{a}	62.86^{a}
PM	0.97^{b}	29.36^{c}	37.40^{de}	0.98^{b}	28.12^{b}	41.00^{cd}
BD	1.11^{b}	31.50^{c}	48.03^{c}	1.11^{b}	30.85^{b}	50.46^{bc}
AG	2.29^{a}	43.56^{a}	62.29^{a}	2.32^{a}	43.35^{a}	67.25^{a}
SEM	0.4	4.0	6.54	0.4	4.7	7.5
			Legumes			
Sheep A						
GS	4.67^{ab}	44.37^{a}	54.33^{a}	5.20^{a}	49.63^{a}	62.40^{a}
LL	2.45^{ab}	23.08^{b}	33.78^{b}	2.53^{b}	22.09^{b}	35.17^{b}
Sheep B						
GS	4.89^{a}	45.73^{a}	55.12^{a}	5.44^{a}	50.68^{a}	63.36^{a}
LL	2.44^{b}	23.49^{b}	33.30^{b}	2.52^{b}	23.02^{b}	35.65^{b}
SEM	0.9	8.3	8.0	1.1	10.5	10.4
			Forage control			
Sheep A						
LP	7.56^{a}	36.86^{a}	41.28	7.87^{a}	38.87^{a}	46.34^{ab}
TA	1.26^{b}	21.60^{b}	38.18	0.92^{b}	20.75^{b}	37.64^{b}
Sheep B						
LP	6.20^{a}	40.22^{a}	42.47	6.49^{a}	41.73^{a}	46.98^{a}
TA	1.32^{b}	25.00^{b}	41.84	1.07^{b}	23.69^{b}	41.74^{ab}
SEM	2.2	5.9	1.7	2.4	7.0	3.1
Pooled SEM	1.5	5.7	6.4	1.6	6.9	7.8

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM, standard error of mean. PP (*P. purpureum*), PM (*P. maximum*), BD (*B. decumbens*), AG (*A. gayanus*), GS (*G. sepium*), LL (*L. leucocephala*), LP (*L. perenne*), TA (*T. aestivum*).

Figure 2 shows the mean effect of A) grasses B) legumes and C) forage controls on tGP (ml/ g OM) and methane (ml/ g digested OM) after 48h of incubation. In general, the forage with the highest tGP recorded the highest methane content. The grasses, legumes and forage controls varied ($P < 0.05$) in tGP and methane content respectively and each forage type tended to follow this order in terms of tGP and methane content: Grasses (AG > PP > BD > PM); legumes (GS > LL); and forage controls (LP > TA).

Figure 3 shows the mean effect of A) grasses B) legumes and C) Forage controls on tGP (ml/ g OM) and methane (ml/ g digested OM) after 72h of incubation. In general, the methane content of each forage type tended to be similar with higher incubation time. The grasses, legumes and forage controls varied ($P < 0.05$) in tGP and methane content respectively and each forage type tended to follow this order in terms of tGP: Grasses (AG > PP > BD > PM); legumes (GS > LL); and Forage controls (LP > TA).

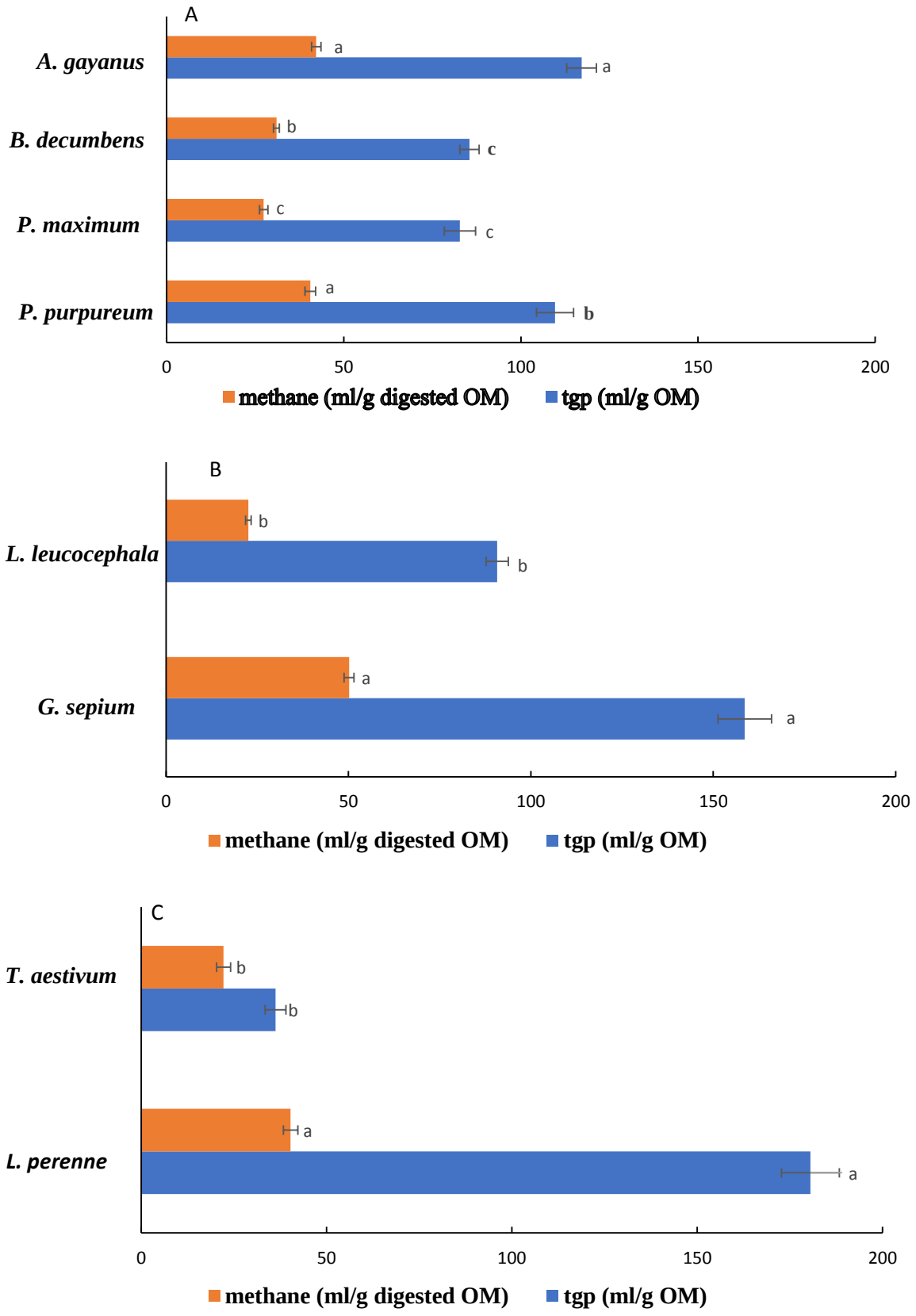


Figure 2 Effect of A) Grasses; B) Legumes; and C) Forage controls on tGP (ml / g OM) and methane (ml / g digested OM) after 48h of incubation

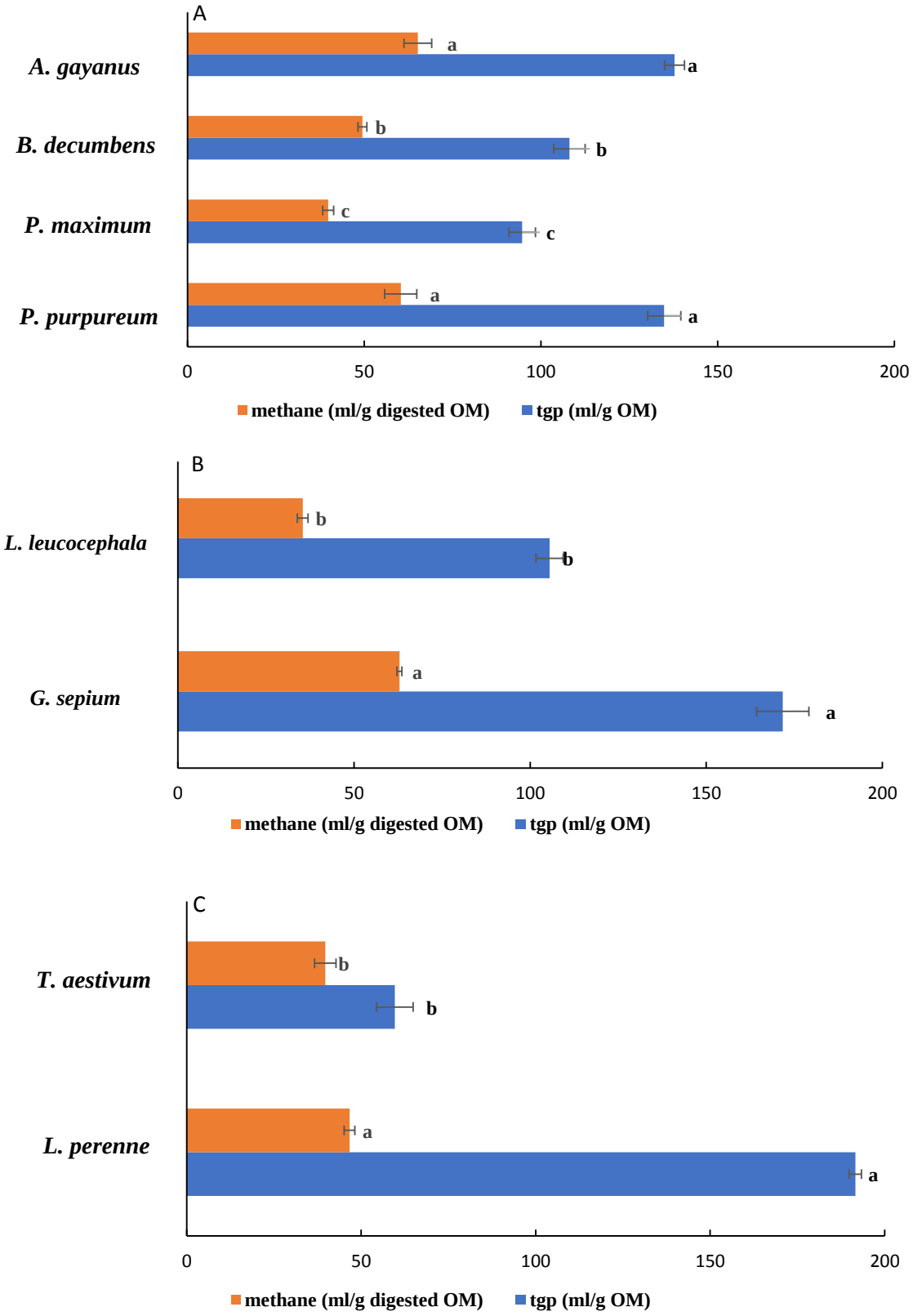


Figure 3 Effect of A) Grasses; B) Legumes; and C) Forage controls on tGP (ml/ g OM) and methane (ml / g digested OM) after 72h of incubation.

Table 18. Shows the main effects of each forage type and sheep RFs on the VFA profiles, pH and NH_3-N concentration obtained after 12h of incubation. The forage types had a significant effect on the VFAs (mmol / L), pH and NH_3-N (mg / L) contents. The Nigerian grasses presented similar acetate, butyrate, isovalerate, and pH. AG presented the highest propionate, valerate, ammonia contents than other grasses. The legumes presented similar valerate and pH, and GS recorded higher acetate, propionate, butyrate, tVFA, ammonia, and A:P contents than LL. The forage controls were different in VFAs composition, and LP recorded higher acetate, propionate, butyrate, valerate, tVFA, ammonia, as well as lower A:P and pH than TA. In most forage types, the sheep RFs had no significant effect on the VFAs fraction, pH and NH_3-N contents. The few exceptions that showed different contents were found in the Nigerian grasses and legumes for acetate, butyrate, valerate and tVFA concentrations.

Table 19 shows the main effect of each forage type and RFs on the VFA profiles, pH and NH_3-N concentration obtained after 48h of incubation. The forage types had a significant effect on the VFAs (mmol / L), pH and NH_3-N (mg / L) contents. The Nigerian grasses presented similar A:P ratio; AG presented the highest acetate, propionate, butyrate, isovalerate, valerate, tVFA, and ammonia contents, as well as lower pH than other grasses. The legumes presented similar pH; GS recorded higher acetate, propionate, butyrate, isovalerate, valerate, tVFA, and ammonia contents, as well as lower A:P ratio than LL. The forage controls were similar in acetate concentration, and LP recorded higher propionate, butyrate, isovalerate, valerate, tVFA, ammonia, as well as the lower A:P and pH than TA. The sheep RFs had no significant effect in most cases on each of the forage type VFAs fraction, pH and NH_3-N contents.

Table 20 shows the mean VFA profiles, pH and NH_3-N for the different forage types and sheep RFs after 72h of incubation. The forages had a significant effect on the VFAs (mmol / L), pH and NH_3-N (mg / L) contents. The Nigerian grasses presented similar acetate, propionate, tVFA, A:P ratio; AG presented the highest butyrate, isovalerate, valerate, and ammonia contents, as well as lower pH than other grasses. The legumes presented similar valerate and pH; GS recorded higher acetate, propionate, butyrate, isovalerate, tVFA, and ammonia contents, as well as lower A:P ratio than LL. The forage controls varied in concentration, and LP recorded higher propionate, butyrate, isovalerate, valerate, tVFA, pH, ammonia, as well as lower acetate concentration and A:P ratio than TA. In most forage types, the sheep RFs had no significant effect on the VFAs fraction, pH and NH_3-N contents.

Table 18 Main effects of each forage type and sheep rumen fluid on VFA (mmol /l) profiles, pH and NH_3-N (mg/l) concentration obtained after 12h of incubation

Main effect of forage type	Acetate	Propionate	Butyrate	Isovalerate	Valerate	TVFA	A:P ratio	pH	NH_3-N
				Grasses					
P. purpureum	42.58	6.23^{a}	1.58	1.08	0.61^{c}	52.07^{ab}	5.37^{a}	6.99	21.26^{b}
P. maximum	39.66	5.00^{b}	1.39	1.09	0.89^{b}	48.55^{b}	6.56^{ab}	7.00	20.02^{b}
B. decumbens	44.73	5.40^{b}	1.61	1.19	1.39^{a}	54.99^{ab}	7.29^{a}	6.94	22.94^{b}
A. gayanus	48.67	6.38^{a}	1.65	1.11	1.51^{a}	59.85^{a}	6.61^{a}	6.99	56.66^{a}
SEM	0.5	0.1	0.01	0.01	0.04	0.6	0.6	0.02	8.2
				Legumes					
G. sepium	76.93^{a}	1.34^{a}	0.33^{a}	1.28^{a}	2.13a	91.88^{a}	8.44	6.95	60.41^{a}
L. leucocephala	57.13^{b}	0.91^{b}	0.21^{b}	0.92^{b}	1.71b	68.50^{b}	7.95	6.99	15.83^{b}
SEM	1.2	0.2	0.04	0.02	0.03	1.5	0.5	0.1	12.0
				Forage control					
L. perenne	67.30^{a}	7.40^{a}	0.57^{a}	1.04	1.42^{a}	112.80^{a}	1.23^{b}	6.74^{b}	36.78^{a}
T. aestivum	28.41^{b}	0.12^{b}	0.06^{b}	1.00	0.19^{b}	34.03^{b}	11.73^{a}	7.00^{a}	7.09^{b}
SEM	2.1	2.0	0.2	0.00	0.1	4.3	2.9	0.1	8.1
Main effect of Sheep									
				Grasses					
A	38.77^{b}	5.29^{b}	1.17	1.00b	1.03^{b}	47.84^{b}	6.28	6.99	29.27
B	49.05^{a}	6.22^{a}	1.95	1.24a	1.17^{a}	59.89^{a}	6.63	6.97	31.70
SEM	0.4	0.1	0.01	0.01	0.03	0.5	0.4	0.02	5.8
				Legumes					
A	59.24^{b}	6.61^{b}	1.89	0.97	1.80^{b}	70.52^{b}	8.92^{a}	7.01	37.23
B	74.82^{a}	9.15^{a}	2.63	1.24	2.03^{a}	89.86^{a}	7.47^{b}	6.93	39.01
SEM	1.2	0.2	0.04	0.02	0.03	1.5	0.5	0.1	12.0
				Forage control					
A	43.55	20.53	1.95	0.90	0.76	67.86	6.55	6.87	20.03
B	52.16	21.62	3.05	1.14	0.85	78.97	6.41	6.87	23.85
SEM	2.1	2.0	0.2	0.00	0.1	4.3	2.9	0.1	8.1

Means with different letters in the same column are significantly different (P<0.05); SEM, standard error of means. A : P; Acetate to Propionate ratio; TVFA: total volatile fatty acids; NH_3-N: ammonia nitrogen.

Table 19 Main effects of each forage type and sheep rumen fluid on the VFA (mmol / l) profiles, pH and NH_3-N (mg/l) concentration obtained after 48h of incubation

Main effect of forage type	Acetate	Propionate	Butyrate	Isovalerate	Valerate	TVFA	A:P ratio	pH	NH_3-N
				Grasses					
P. purpureum	88.59^{ab}	52.53^{b}	7.44^{ab}	0.14^{b}	1.60^{ab}	150.34^{b}	1.41	6.76^{b}	26.68^{b}
P. maximum	71.84^{b}	44.69^{b}	3.08^{c}	0.70^{a}	1.20^{b}	121.51^{c}	1.26	6.88^{a}	22.59^{b}
B. decumbens	75.38^{b}	48.46^{b}	6.11^{b}	0.67^{a}	1.67^{ab}	132.21^{bc}	1.23	6.79^{b}	26.03^{b}
A. gayanus	104.13^{a}	71.15^{a}	7.77^{a}	0.93^{a}	2.15^{a}	186.13^{a}	1.24	6.74^{c}	68.44^{a}
SEM	1.5	1.1	0.3	0.02	0.04	2.7	0.1	0.03	10.0
				Legumes					
G. sepium	142.82^{a}	24.79^{a}	6.45^{a}	1.63^{a}	2.60^{a}	178.28^{a}	6.05^{b}	6.88	79.33^{a}
L. leucocephala	107.64^{b}	15.85^{b}	3.36^{b}	0.85^{b}	2.13^{b}	129.83^{b}	7.80^{a}	6.87	32.89^{b}
SEM	2.2	0.5	0.2	0.1	0.03	3.0	0.5	0.03	12.7
				Forage control					
L. perenne	111.01	79.96^{a}	10.80^{a}	2.00^{a}	5.75^{a}	209.52^{a}	1.19^{b}	6.65^{b}	75.20^{a}
T. aestivum	102.67	16.67^{b}	2.66^{b}	0.48^{b}	0.54^{b}	123.02^{b}	7.02^{a}	6.85^{a}	12.03^{b}
SEM	1.0	3.4	0.4	0.1	0.3	4.8	1.6	0.1	16.9
Main effect of sheep									
				Grasses					
A	84.38	53.20	4.76^{b}	0.63	1.60	144.58	1.30	6.80	34.21
B	85.59	55.21	7.44^{a}	0.59	1.68	150.51	1.27	6.77	37.66
SEM	1.0	0.8	0.2	0.02	0.03	1.9	0.1	0.02	7.0
				Legumes					
A	115.99^{b}	18.58^{b}	4.76	1.05^{b}	2.31	242.69^{b}	7.13	6.88	54.05
B	134.47^{a}	22.06^{a}	5.04	1.42^{a}	2.42	165.42^{a}	6.72	6.87	58.16
SEM	2.2	0.5	0.2	0.1	0.03	3.0	0.5	0.03	12.7
				Forage control					
A	102.80	46.36	6.10^{b}	1.21	3.11	159.58	4.25	6.77	43.14
B	110.88	50.27	7.37^{a}	1.26	3.18	172.96	3.96	6.73	44.09
SEM	1.0	3.4	0.4	0.1	0.3	4.8	1.6	0.1	16.9

Means with different letters in the same column are significantly different ($P<0.05$); SEM, standard error of means. A : P; Acetate to Propionate ratio. TVFA: total volatile fatty acids; NH_3-N: ammonia nitrogen.

Table 20 Main effects of each forage type and sheep rumen fluid on the VFA (mmol / l) profiles, pH and NH_3-N (mg/l) concentration obtained after 72h of incubation

Main effect of forage type	Acetate	Propionate	Butyrate	Isovalerate	Valerate	TVFA	A:P ratio	Ph	NH_3-N
				Grasses					
P. purpureum	101.43	74.80	7.68^{ab}	0.30^{b}	1.45^{b}	185.66	1.08	6.70^{ab}	39.43^{b}
P. maximum	93.91	62.55	4.82^{c}	0.76^{a}	1.15^{c}	163.19	1.18	6.82^{a}	31.97^{b}
B. decumbens	99.06	68.87	6.96^{b}	0.70^{a}	2.25^{a}	177.84	1.15	6.76^{ab}	33.99^{b}
A. gayanus	109.61	76.83	8.12^{a}	0.97^{a}	2.30^{a}	197.83	1.16	6.66^{b}	75.95^{a}
SEM	1.1	1.0	0.2	0.02	0.1	2.2	0.03	0.04	9.4
				Legumes					
G. sepium	155.12^{a}	26.95^{a}	7.93^{a}	1.73^{a}	2.77	194.49^{a}	5.53^{b}	6.82	90.27^{a}
L. leucocephala	117.95^{b}	15.82^{b}	4.21^{b}	0.89^{b}	2.08	140.95^{b}	7.44^{a}	6.85	44.24^{b}
SEM	2.1	0.6	0.2	0.1	0.1	3.0	0.5	0.02	12.4
				Forage control					
L. perenne	114.50^{b}	83.26^{a}	18.77^{a}	2.01^{a}	6.26^{a}	217.91^{a}	1.13^{b}	6.64^{b}	86.75^{a}
T. aestivum	128.14^{a}	23.64^{b}	7.53^{b}	0.25^{b}	1.00^{b}	160.56^{b}	5.10^{a}	6.76^{ab}	17.04^{b}
SEM	0.9	3.2	0.3	0.1	0.3	3.2	1.1	0.03	18.7
Main effects of sheep									
				Grasses					
A	96.22	68.64	5.66^{b}	0.73	1.72	172.97	1.15	6.74	43.91
B	105.79	72.88	8.13^{a}	0.64	1.86	189.29	1.14	6.72	46.75
SEM	0.8	0.7	0.1	0.02	0.04	1.6	0.02	0.03	6.6
				Legumes					
A	131.40	19.96	5.50^{b}	1.12^{b}	2.32	160.30	6.70^{a}	6.84	65.15
B	141.67	22.81	6.63^{a}	1.50^{a}	2.52	175.13	6.27^{b}	6.82	69.36
SEM	2.1	0.6	0.2	0.1	0.1	3.0	0.5	0.02	12.4
				Forage control					
A	115.31	51.32	8.56	1.23^{a}	3.54	198.50	3.17	6.71	51.26
B	127.33	55.58	10.84	1.03^{b}	3.72	179.97	3.06	6.69	52.53
SEM	0.9	3.2	0.3	0.1	0.3	3.2	1.1	0.03	18.7

Means with different letters in the same column are significantly different ($P<0.05$); SEM, standard error of means. A : P; Acetate to Propionate ratio TVFA: total volatile fatty acids; NH_3-N: ammonia nitrogen.

Table 21 shows the gas production constants of different forage types incubated in buffered rumen fluid. In general, the highest lag phase, rate of gas production and potential of gas yield were recorded in TA (22.77h), LP (7.74 ml / g OM) and LP (187.8 ml/ g OM) respectively. Among the Nigerian grasses, the lag phase followed this order: PM > BD > AG > PP, while the rate of gas production and potential yield of gas production followed this order: AG > PP > BD > PM. Among the legumes, GS recorded higher lag phase, rate of gas production and potential yield of gas than LL. Among forage controls, LP recorded lower lag phase (i.e. around 4x lower) but higher rate of gas production (i.e. around 5x higher) and potential yield of gas production (i.e. around 2x higher) than TA.

Table 21 Gas production constants (ml / g OM) of different forage types incubated in buffered rumen fluid.

Forages	**L (hours)**	**U (ml / g OM)**	**a (ml /g OM)**
		Grasses	
PP	15.12	3.88	142.2
PM	18.67	3.28	96.0 2
BD	18.13	3.15	110.4
AG	16.53	3.97	147.7
		Legumes	
GS	7.14	5.11	175.6
LL	4.93	2.25	114.7
		Forage control	
LP	5.28	7.74	187.8
TA	22.77	1.37	93.01

L: lag phase (hours); U: rate of maximum gas production (ml / g OM); a: potential of gas yield (ml / g OM)

Table 22 shows the correlation coefficients (r) between proximate, fibre and secondary metabolite compositions. The CP was negatively correlated to fibre components ($P < 0.01$), hemicellulose and cellulose ($P < 001$), and positively correlated to secondary metabolite contents ($P < 0.01$). A strong positive correlation was recorded between each of the fibre components and their corresponding cellulose and hemicellulose contents. The fibre components and the corresponding cellulose content were negatively correlated to the secondary metabolite contents. The secondary metabolite contents and TAC were strongly positively correlated to each other ($P < 0.001$).

Table 23 shows the correlation coefficients between chemical composition (g / kg DM), secondary metabolites (g / kg D), *in vitro* gas production characteristics, *in vitro* digestibility of organic matter and ammonia-nitrogen concentration (mg / l) of the forage types. The gas production at 12h, 48h and 72h were negatively correlated to fibre and lignin contents as well as their corresponding cellulose and hemicellulose, and weakly positively correlated to CP. The lag phase (i.e. hours spent before producing gas) was negatively correlated to CP and secondary metabolites, and strongly positively correlated to fibre and lignin contents and their corresponding hemicellulose and cellulose. The rate of gas produced and potential of gas yields were negatively correlated to fibre and lignin contents and their corresponding cellulose and hemicellulose, as well as secondary metabolites, and positively correlated to CP. The IVOMD and ammonia at 12h, 48h and 72h of incubation were positively correlated to CP and negatively correlated to fibre and lignin contents and their corresponding hemicellulose and cellulose content.

Table 24 shows the correlation coefficients between gas production characteristics, *in vitro* organic matter degradability (g / kg DM), ammonia-nitrogen (mg / l), and methane (ml / g digested OM). The potential of gas production was positively correlated to the rate of gas production ($P < 0.001$), and negatively correlated to the lagphase ($P > 0.05$). The total gas production at 12h, 48h and 72h of incubation were negatively correlated to lag phase, and positively correlated to the rate of gas production and the potential of gas yield. The IVOMD and ammonia content at 12h, 48h and 72h were negatively correlated to lagphase, and strongly positively correlated to rate of gas production, potential of gas yield and total gas production at 12h, 48h and 72h respectively. The NH_3-N contents at 12h, 48h and 72h were positively correlated to IVOMD at 12h, 48h, and 72h respectively. Methane content (12h) was negatively correlated to lagphase; and strongly positively correlated to the rate of gas production, potential of gas yield, total gas production and IVOMD.

Table 25 shows the correlation coefficients between gas production characteristics, total gas production (ml / g OM), NH_3-N (mg / l) , *in vitro* organic matter degradability (g / kg DM) and volatile fatty acid (mmol / l) production for forage types incubated in buffered rumen fluid for 12h, 48h and 72h. Total VFAs concentration and each of its fractions (i.e. acetate, propionate, butyrate, and valerate) at 12h, 48h and 72h of incubation were negatively correlated to lag phase, and positively correlated to the rate of gas production, potential of gas yield, total gas production, NH_3-N concentration, IVOMD and methane concentration respectively.

Table 22 Correlation coefficients (r) for relationships between proximate, fibre and secondary metabolite compositions of the forage types

Predictors	**NDF**	**ADF**	**ADL**	**Hem**	**Cell**	**TP**	**TT**	**CT**	**HT**	**TA**
CP	-0.91**	-0.94***	0.2	-0.61	-0.93***	0.82**	0.81**	0.85**	0.69	0.68
NDF	-	0.98***	-0.003	0.81**	0.91**	-0.74*	-0.72*	-0.75*	-0.62	-0.64
ADF	-	-	-0.11	0.69	0.96***	-0.80*	-0.79*	-0.82**	-0.66	-0.68
ADL	-	-	-	0.34	-0.4	0.04	0.06	0.12	-0.09	-0.11
HEM	-	-	-	-	0.53	-0.37	-0.35	-0.35	0.32	-0.35
CEL	-	-	-	-	-	-0.75*	-0.74*	-0.79*	-0.59	-0.6
TP	-	-	-	-	-	-	1.00***	1.00***	0.97***	0.97***
TT	-	-	-	-	-	-	-	1.00***	0.98***	0.97***
CT	-	-	-	-	-	-	-	-	0.95***	0.95***
HT	-	-	-	-	-	-	-	-	-	1.00***

CP; Crude protein, NDF; Neutral detergent fibre, ADF; Acid detergent fibre, ADL; Acid detergent lignin, Hem; hemicellulose; Cell; cellulose, TP; total phenols, TT; total tannins, CT; condensed tannins, HT; hydrolysable tannins, TA; total antioxidants.

* $P < 0.05$

**$P < 0.01$

***$P < 0.001$

Table 23 Correlation coefficients (r) for relationships between chemical composition (g / kg DM), secondary metabolites (g / kg DM), in vitro gas production characteristics, in vitro digestibility of organic matter and ammonia-nitrogen concentration (mg / l) of the forage types

	Gas production (ml/g OM)			**Gas production constants**			**IVOMD g/ kg DM)**			**Ammonia (mg / l)**		
Predictor	12h	48h	72h	L (hours)	U (ml/g OM)	a (ml/g OM)	12h	48h	72h	12h	48h	72h
CP	0.59	0.46	0.42	-0.85**	0.19	0.42	0.780*	0.40	0.36	0.42	0.53	0.56
NDF	-0.81*	-0.56	-0.51	0.95***	-0.39	-0.49	-0.89**	-0.58	-0.52	-0.30	-0.56	-0.59
ADF	-0.72*	-0.46	-0.41	0.91**	-0.26	-0.40	-0.83**	-0.46	-0.42	-0.23	-0.46	-0.50
ADL	-0.16	-0.29	-0.32	0.18	-0.41	-0.25	-0.15	-0.21	-0.27	0.15	-0.05	-0.07
Hem	-0.91**	-0.76*	-0.71*	0.88**	-0.73*	-0.67	-0.89**	-0.82*	-0.74*	-0.44	-0.74*	-0.76*
Cell	-0.61	-0.34	-0.28	0.78*	-0.11	-0.29	-0.72*	-0.35	-0.30	-0.26	-0.41	-0.44
TP	0.22	-0.01	-0.05	-0.62	-0.24	-0.07	0.44	-0.08	-0.12	-0.11	0.02	0.05
TT	0.19	-0.04	-0.07	-0.59	-0.27	-0.08	0.41	-0.11	-0.14	-0.11	0.01	0.04
CT	0.23	-0.04	-0.03	-0.62	-0.25	-0.04	0.45	-0.07	-0.10	-0.06	0.05	0.08
HT	0.08	-0.14	-0.17	-0.50	-0.33	-0.19	0.30	-0.21	-0.24	-0.23	-0.10	-0.08
TA	0.12	-0.13	-0.16	-0.51	-0.30	-0.19	0.32	-0.18	-0.22	-0.27	-0.11	-0.09

CP; Crude protein, NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; Hem: hemicellulose; Cell: cellulose; TP: total phenols; TT: total tannins; CT: condensed tannins; HT: hydrolysable tannins; TA: total antioxidants; L: lag phase (hours); U: rate of maximum gas production (ml / g OM); a: potential of gas yield (ml / g OM); IVOMD: in vitro organic matter digestibility.

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 24 Correlation coefficients (r) for relationships between gas production characteristics, in vitro organic matter degradability (g / kg DM), ammonia (mg / l), and methane (ml / g digested OM).

	Gas production constants			**Total gas production (ml /g OM)**			**IVOMD (g / kg DM)**			**Ammonia (mg / l)**			**CH_4**
Predictors	L (hour)	U (ml/g OM)	a (ml/g OM)	12h	48h	72h	12h	48h	72h	12h	48h	72h	12h
L	-	-0.59	-0.69	-0.88**	-0.75*	-0.72*	-0.96***	-0.75*	-0.70*	-0.39	-0.65	-0.69	-0.80*
U	-	-	0.90**	0.82**	0.94***	0.94***	0.72*	0.96***	0.96***	0.60	0.80*	0.82**	0.86**
A	-	-	-	0.83**	0.96***	0.97***	0.76*	0.96***	0.98***	0.76*	0.91**	0.93***	0.86**
TGP- 12h	-	-	-	-	0.87**	0.83**	0.92***	0.93**	0.90**	0.48	0.77*	0.80*	0.99***
TGP - 48h	-	-	-	-	-	0.99***	0.85**	0.98***	0.97***	0.74*	0.90**	0.93***	0.87**
TGP - 72h	-	-	-	-	-	-	0.82*	0.96***	0.97***	0.75*	0.89**	0.92***	0.84*
IVOMD- 12h	-	-	-	-	-	-	-	0.86**	0.79*	0.51	0.75*	0.79*	0.86**
IVOMD- 48h	-	-	-	-	-	-	-	-	0.99***	0.65	0.87**	0.90**	0.95***
IVOMD- 72h	-	-	-	-	-	-	-	-	-	0.68	0.88**	0.90**	0.93***

L: lag phase (hours); U: rate of maximum gas production (ml / g OM); a: potential of gas yield (ml / g OM); TGP: total gas production; IVOMD: in vitro organic matter degradability; CH_4: methane (ml / g digested OM).

* $P < 0.05$

**$P < 0.01$

***$P < 0.001$

Table 25 Correlation coefficients (r) for relationship between gas production characteristics, total gas production (ml / g OM), ammonia (NH_3-N; mg / l) , in vitro organic matter degradability (g / kg DM), methane (CH_4; ml / g OM) and volatile fatty acid (mmol / l) production for forage types incubated in buffered rumen fluid for 12h, 48h and 72h.

	Total VFA			**Acetate**			**Propionate**			**Butyrate**			**Valerate**		
Predictors	12h	48h	72h	12h	48h	72h	12h	48h	72h	12h	48h	72h	12h	48h	72h
L	-0.86**	-0.54	-0.24	-0.89**	-0.62	-0.40	-0.59	-0.06	0.15	-0.80*	-0.43	-0.22	-0.74*	-0.74*	-0.67
U	0.89**	0.88**	0.88**	0.72*	0.36	0.13	0.87**	0.70*	0.55	0.90**	0.88**	0.78*	0.44	0.92***	0.89**
A	0.88**	0.95***	0.85**	0.82**	0.66	0.42	0.71*	0.52	0.33	0.84**	0.87**	0.76*	0.57	0.83**	0.78*
TGP - 12h	0.98***	0.74*	0.55	0.89**	0.68	0.49	0.83**	0.24	0.01	0.97***	0.61	0.58	0.63	0.90**	0.88**
TGP - 48h	0.93***	0.89**	0.80*	0.88**	0.55	0.29	0.75*	0.55	0.38	0.88**	0.84**	0.65*	0.65	0.87**	0.82**
TGP - 72h	0.90**	0.91**	0.83**	0.85**	0.54	0.28	0.72*	0.58	0.42	0.85**	0.88**	0.68	0.62	0.86**	0.80*
NH_3N- 12h	0.58	0.78*	0.69	0.70*	0.61	0.44	0.24	0.4	0.23	0.45	0.59	0.43	0.71*	0.44	0.41
NH_3N- 48h	0.84**	0.92***	0.76*	0.85**	0.72*	0.49	0.59	0.46	0.22	0.76*	0.72*	0.61	0.75*	0.75*	0.72*
NH_3N- 72H	0.87**	0.92***	0.75*	0.88**	0.71*	0.47	0.6	0.46	0.22	0.78*	0.74*	0.6	0.76*	0.77*	0.73*
IVOMD 12h	0.94***	0.62	0.39	0.96***	0.60	0.39	0.65	0.17	0.03	0.86**	0.51	0.30	0.80*	0.80*	0.74*
IVOMD 48h	0.97***	0.89**	0.77*	0.87**	0.56	0.30	0.83**	0.52	0.35	0.95***	0.80*	0.69	0.59	0.92***	0.88**
IVOMD 72h	0.93***	0.92***	0.85**	0.83**	0.61	0.38	0.81**	0.53	0.35	0.92***	0.86**	0.79*	0.55	0.90**	0.87**
CH_4 12h	0.97***	0.80*	0.64	0.86**	0.70	0.52	0.85**	0.29	0.06	0.97***	0.65	0.68	0.58	0.91**	0.89**
CH_4 48h	0.59	0.80*	0.83**	0.66	0.53	0.36	0.33	0.48	0.39	0.50	0.75*	0.59	0.49	0.48	0.43

L: lag phase (hours); U: rate of maximum gas production (ml / g OM); a: potential of gas yield (ml / g OM); TGP: total gas production; NH_3N: ammonia nitrogen (mg / l); IVOMD: in vitro organic matter degradability; CH_4: methane (ml / g digested OM).

* $P < 0.05$

**$P < 0.01$

***$P < 0.001$

2.7 Discussion

The aim of this study was divided into three aspects. Firstly, to ascertain the nutritive quality of the Nigerian grasses and select two out of the four grasses for further biological improvement. The grasses selected are those with the lowest nutrient composition (i.e low in CP and EE, high in fibre and lignin contents), lowest nutrient utilisation (i.e low in nutrient degradability, low in ammonia content, low in volatile fatty acids, and high in methane production), and highest ability to support microbial growth i.e. a criteria needed in forages to promote biological upgrade (as discussed further in Chapter 3). The grasses were collected during the dry season and the hypothesis is that they are expected to be of low nutritive quality, i.e. they should be of poor nutrient composition and not easily degraded or fermented in the rumen due to their high structural components such as cell wall. This prior information formed the basis for using longer incubation time and the justification for our evaluation and selection of grasses in the *in vitro* study and microbial study (see Chapter 3). Secondly, to ascertain the nutritive quality of two widely cultivated legumes before testing one legume for its use as a complementary feed with the grasses chosen in order to enhance feed utilisation, reduce enteric methane production, increase rumen protein bypass, and boost the immune system of ruminant animals. The selected legume should be high in soluble fraction (i.e high in CP and EE), low in fibre (ADF and NDF), high in lignin (i.e a property of tropical woody legume collected during the dry season) and high in secondary metabolites and TAC. It should also limit methane production, reduce excessive degradation of protein to ammonia content in the rumen, and support rumen microbial growth. Thirdly, to evaluate the nutrient quality of two contrasting forage controls for their use as positive and negative controls for the Nigerian grasses, with or without a legume.

Almost all Nigerian grasses appeared to be of low nutritive quality and thus the use of longer incubation times for the *in vitro* study was justified. Most of the chemical contents of those grasses remained within the expected levels, e.g. CP < 80 g / kg DM (Narmsilee *et al.*, 2002; Titgemeyer *et al.*, 2004), EE < 20 g / kg DM (Harfoot and Hazlewood, 1997), NDF > 650 g / kg DM and ADF > 450 g / kg DM (Van Saun, 2006), as previously reported for similiar low quality forages. Although AG recorded higher CP content, it also contained fibre and lignin contents that were even higher than some other grasses. The high CP content supports the report of CIAT (1978), that it contains higher nitrogen content along with high fibre content. AG accumulated higher secondary metabolites, showing that there was a possibility that this grass could cause a reductive effect on the rumen microbial community due to its higher TT content when compared to other grasses. The higher secondary metabolites in AG supports the findings of Ogungbesan *et al.* (2011), although the high tannin content was unusual. In general, the

grasses, based on their chemical contents, can be grouped into two subclasses of low quality i.e. low (AG and PP) and very low (PM and BD).

The grasses have shown that their nutrients are poorly utilised in the rumen. They had low nutrient digestibility (i.e. IVDMD and IVOMD), low ammonia-nitrogen content, high total volatile fatty acids based on increase in acetate content, and high methane content. Their nutrient digestibility was less than 550 g / kg (Leng, 1990) found in most low-quality tropical grasses, which is a reflection of their high fibre and lignin contents that limited their digestibility (Newman *et al.*, 2006). The differences obtained in the nutrient degradability of the grasses might be linked with their chemical composition, in particular the CP, fibre, lignin and mineral contents, and secondary metabolites, as well as forage species. Their ammonia-nitrogen content was below the values of 50 - 200 mg / L reported as the critical amount required for efficient fermentation of fibre by rumen microbes on high quality and low quality forages respectively (Satter and Slyter, 1974; Boniface *et al.*, 1986; Perdok and Leng, 1989; Nguyen *et al.*, 2016; Tomkins *et al.*, 2018). In addition to this, it has been reported that the value of 62 and 214 mg / L is regarded as an optimal NH_3-N concentration for microbial growth and multiplication when diets with CP levels that were higher and lower than 6% were fed, respectively (Hoover, 1986). The higher NH_3-N concentration of AG can be attributed to its higher CP content, as CP was found to be positively correlated with ammonia-nitrogen concentration (NRC, 1996). Also, highly fermented substrates are known to produce a higher amount of microbial protein from which ammonia is generated (Cone and Becker, 2012) at an earlier incubation time than slowly fermented substrates, after which the substrates are exhausted by microbes. Due to the exhaustion of nutrients in the substrates, the ammonia-nitrogen generation from easily degraded forages is slowed down, of which that of the slowly degraded substrates is moving up as the substrates are not yet exhausted (Bach *et al.*, 2005). Despite moderate ammonia-nitrogen concentration for AG, none of the grasses used in this study were capable of meeting the ammonia-nitrogen concentration required for an efficient nutrient degradability. Therefore, these forages were regarded as low quality because they would need to be supplemented with sources of rumen degradable protein.

The pH values obtained for all the grasses were beyond 6.5, which was perhaps due to the presence of cellulose-degrading microbes (Nagaraja and Titgemeyer, 2007) that were needed to support fibre digestion. The similar high pH of the grasses was an indication that they contained more structural carbohydrates than soluble carbohydrates, that are known to cause a rapid drop in the rumen pH (Slyter, 1976; France and Dijkstra, 2005; Nagaraja and Titgemeyer, 2007).

The highest tGP, rate of gas production and potential of gas yield of AG is an indication that its nutrients were degraded more or fermented at a faster rate, as there was a close relationship between feed degradability and the amount of gas obtained when feed was incubated with rumen fluid *in vitro* (Menke *et al.*, 1979). The AG and PP nutrients were degraded by rumen microbes at a faster rate than BD and PM, which was reflected in their respective tGP, rate of gas production and potential of gas yields. Their increase might be because they contained more CP and EE contents than the other grasses, as CP and EE are positively correlated to better fermentation of feed (Newman *et al.*, 2006; Hariadi and Santoso, 2010). However, despite a higher rate of gas production of AG, the expectation was for it to maximize gas production at initial hours of incubation like PP but this was not the case. In general, all of the grasses required long hours for gas production to be maximized, but AG and BD needed more extended hours (i.e. lag phase) than other grasses. This indicates that these grasses were still supporting the growth and degradation activity of microbes after a longer period of incubation (i.e. 48 and 72hours). It also showed that these grasses contained more structural carbohydrates than other grasses, which requires longer retention time for more nutrients to be degraded by cellulolytic microbes (Ho and Abdullah, 1999), thus making a longer incubation time suitable for microbial investigation (Chapter 3).

The grasses produced high methane and less tVFAs, and this supports the findings of Kim *et al.* (2013) in an *in vitro* experiment where different feeds were evaluated for their potential to generate methane and change methanogen diversity. In their research, it was discovered that highly structural feeds, i.e. LQF, tend to produce higher methane and lower VFAs. However, the highest methane content of AG indicates the presence of highly structural carbohydrate (i.e. fibre content) and high protein content. The protein supplied the methanogens with the required nitrogen needed for their activity in fermenting the substrate. However, due to their fibrous nature, the fermentation time became longer and subsequently allowed more methane production (Van Kessel and Russell, 1996). This, therefore, qualifies AG, despite its high CP content, as a low quality grass just like other grasses.

tVFA concentrations of all the grasses at an early hour of incubation were not capable of meeting the tVFA (i.e acetate to butyrate) production ranges of 80 -16 mmol/l required for optimum microbial growth (Van Soest, 1994) and the VFA ranges of 60 mM, 20mM and 10mM for acetic acid, propionate and butyrate respectively in the rumen (Madigan *et al.*, 2000) when on good quality forage. This indicates that they were not good quality forages. Also, the greater amount recorded at later hour, was based on the increase in the acetate molar fraction of the tVFA,which at the long run may not be beneficial to the animal as rumen microbes especially

bacteria and fungi utilises it for methane production (Bernalier *et al.*, 1991; Ho and Abdullah, 1999; Kamalak *et al.*, 2002). Indeed, they need to be upgraded biologically so that the locked carbohydrates can be accessible for further microbial degradation in the rumen, thus releasing the required nutrients for animal growth and production. The highest branched-chain VFAs (valeric and isovaleric acids) concentration of AG was as a result of its higher CP content and ammonia concentration. This is because, during substrate fermentation in the rumen, amino acid deamination and further breakdown of the amino acid carbon skeleton leads to the production of NH_3 and branched chain VFAs, respectively (Chalupa, 1976; France and Dijkstra, 2005). In general, grouping the Nigeria grasses with respect to the nutrient fermentation profiles gives these 2 categories: 1) low-quality (AG and PP), and 2) very low-quality (BD and PM), but if methane is to be included they can be grouped as follows: 1) low quality (PP and PM), and very low-quality (AG and BD). This evaluation gave a clue as to which grasses could be selected for their feeding to ruminants. However, it was difficult to make the selection based on the outcome of this evaluation alone. Thus the microbial composition of the *in vitro* degraded forages was examined in Chapter 3 as a further criterion before the selection of suitable grasses. Also, it has been found that *in vitro* gas production was just an indirect method of measuring substrate degradation, especially carbohydrate i.e. VFAs, and in most cases (i.e. in straws) not positively correlated with the creation of microbial biomass (Blümmel *et al.*, 1997; Liu *et al.*, 2002).

The legumes have shown that they can be used as a complementay protein feed, due to their high protein and ammonia concentration, and not as an energy complementary feed, due to their low tVFAs production in the rumen. In terms of their ability to reduce enteric methane production, only LL was capable of achieving this purpose. Also, it showed the highest TAC which suggested that it might be capable of boosting the immune system (Chew, 1995; Weiss, 2005). The legumes, despite being collected during the harsh weather, can be classified as high-quality forages due to their higher CP and EE contents, as well as lower fibre contents. This is a reflection that the digestible fractions of feed in legumes are a bit more stable, even with the effect of harsh weather, maturity or age of regrowth (Giovanni, 1990; Ayres *et al.*, 1998). Higher CP and EE contents in the feed are indicators that the feed is capable of increasing the ruminant feed intake and productivity through better digestibility (France *et al.*, 2000; Fraser *et al.*, 2004). Therefore, there is the possibility that they can be used as supplements to improve the utilisation of low-quality forages. The higher metabolites accumulated by these legumes support the findings that most legumes contain higher polyphenols, which are regarded as secondary metabolites (Cudjoe and Mlambo, 2014; Franzel *et al.*, 2014). The TT content of LL

was higher than the threshold levels (50 g / kg) reported by Chesson *et al.* (1982) to inhibit the growth and activity of specific microbes, such as bacteria and anaerobic fungi (Akin and Rigsby, 1985; Patra and Saxena, 2011; Goel and Makkar, 2012), that are involved in the degradability of fibre and which directly or indirectly reduce the feed intake (Patra and Saxena, 2011; Bhatta *et al.*, 2013b). There is the possibility that the legumes, in particular LL, are capable of reducing the enteric methane production and/or use as a supplement in maintaining animal health by boosting the immune system due to higher TAC (Roeder, 1995).

The legumes response to nutrient utilisation by rumen microbes was different. GS nutrient degradability was within the range of 612 – 696g /kg (Chapman, 1985) reported for good quality forages, while LL had less than 550 g / kg degradability as found in low-quality forages (Leng, 1990). The differences between the legumes can be due to their different secondary metabolites (i.e. tannin) contents, as it was evident that secondary metabolites do negatively affect forage fermentation and nutrient degradability (Guglielmelli *et al.*, 2011; Sebata *et al.*, 2011). LL did exhibit this reductive effect on nutrient degradability but GS did not. The higher nutrient degradability of GS indicates that tannins are either present in an amount that is not capable of altering the rumen microbes fermentative ability, or that the tannins have been transformed to a non-toxic compound by the rumen microbes. The lower nutrient degradability obtained in LL supports the fact that tannin tends to exhibit a toxic effect on rumen microbes (Bhatta *et al.*, 2009) that are involved in fibre fermentation, thus depressing fermentation and digestion. Therefore, it has shown itself to be a better choice in altering the rumen microbes' fermentative ability and that the metabolites were not transformed to a non-toxic compound by the rumen microbes. The higher NH_3-N concentration of GS is far more than the 50 mg/ L reported by NRC (1988) as the minimal NH_3-N concentration required for an efficient nutrient digestion by rumen microbes. This indicates that GS contained a high amount of easily degradable protein (Hume, 1970b; Nagaraja and Titgemeyer, 2007) that led to the accumulation of a higher amount of ammonia-nitrogen in the rumen. The feeding of animals with such feeds producing higher ammonia-nitrogen is wasteful and not profitable (Roffler and Thacker, 1983; Kalscheur *et al.*, 1999), and it causes reduced nitrogen utilisation efficiency (Castillot *et al.*, 2000).The lower NH_3-N concentration obtained in LL is an indication that a protein-tannin complex was formed which prevented nitrogen from being degraded to ammonia by rumen microbes (Bhatta *et al.*, 2009), thus altering the synthesis of microbial protein (Bhatta *et al.*, 2009; Grainger *et al.*, 2009). This makes LL a better choice as a possible feed that facilitates better protein by pass in the rumen and can be used to supplement low quality forages for ruminants.

The pH ranges obtained in this study were beyond 6.5 showing that this was perhaps due to the presence of cellulose degrading microbes (Nagaraja and Titgemeyer, 2007) that supported fibre digestion and consequently the rise in pH. The pH of the legumes was expected to be lower than what was recorded, as they contained higher soluble carbohydrates that aid in the secretion of organic acids, the major cause of pH fall (Ho and Abdullah, 1999; Krishna, 2005).This might be due to their high secondary metabolites (Suharti *et al.*, 2011), which might have altered the microbial composition by reducing the activity of some soluble carbohydrate utilising microbes (Anantasook *et al.*, 2013; Pilajun and Wanapat, 2013).

The highest tGP, rate of gas production and potential of gas yield of GS is an indication that its nutrients are more degraded or fermented at a faster rate compared to LL, as there was a close positive relationship between feed degradability and the amount of gas obtained when feeds were incubated with rumen fluid in an *in vitro* study (Menke *et al.*, 1979). LL was expected to produce higher tGP, the rate of gas production and potential of gas production, respectively, as it contained more CP and EE content than GS; these are usually positively correlated to fermentation (Newman *et al.*, 2006; Hariadi and Santoso, 2010), however, this was not the case. This indicates that its higher secondary metabolites contents might have altered the rumen fermentation pathway by exhibiting a toxic effect on the rumen microbes, especially bacteria that are involved in the degradation process (Bhatta *et al.*, 2009; Wang *et al.*, 2009; Mao *et al.*, 2010; Anantasook *et al.*, 2013). It might also be that it formed tannin-carbohydrate and tannin-protein complexes that prevented the degradation of carbohydrates and protein in the rumen, thus leading to a reduced tGP (Mueller-Harvey, 2006; Bhatta *et al.*, 2009; Patra and Saxena, 2011) and increased by-pass CP from the rumen. Metabolites are known to exhibit antimicrobial characteristics (Tavendale *et al.*, 2005; Patra and Saxena, 2011; Goel and Makkar, 2012) that limit the growth and survival of certain bacteria in the rumen. The longer hours (i.e. lag phase) required for LL to maximize its gas production is an indication that the metabolites were reducing the activities of ruminal microbes involved in the degradability of the forages. This makes LL a possible forage for altering the ruminal fermentation pathway to increase the amount of nutrients (i.e. protein) bypass from the rumen to abomasum for their efficient utilisation by the animal.

LL produced less methane gas and total VFAs than GS, even though it contained high CP content and higher structural carbohydrates. This supports the findings that most tannin containing tropical legumes tend to reduce methane production in the rumen (Bhatta *et al.*, 2009; Mao *et al.*, 2010) through reduced fibre digestion (Tiemann *et al.*, 2008). They also reduce plant protein degradation to ammonia-nitrogenn by forming tannin-protein complexes,

thus inhibiting microbial growth and multiplication (Tavendale *et al.*, 2005) and increasing N turnover from the rumen into abomasum. This was not the case with GS, as it produced twice the amount of methane produced by LL. This may be due to the fact that it was more fibrous due to maturity (McCaughey *et al.*, 1997; Benchaar *et al.*, 2001), and since it has been found that legume fibre was more lignified and not easily digestible by microbes when compared with grass fibre (Buxton and Redfearn, 1997). The longer time spent by the microbes in trying to digest the fibre has created more time for them in utilising the contained higher protein content for their methanogenic activity. This, therefore, makes LL a better choice for reducing methane production in the rumen.

The tVFAs concentration of the legumes is not capable of meeting the tVFA production range (80 -16mmol/l) required for optimum microbial growth (Van Soest, 1994) when on good quality forage. The acetate production of GS was more than the needed acetate content of 20mM. This might have been the reason why it produced higher methane content, as higher acetate concentration has been found to be proportional to methane production (Kamalak *et al.*, 2002). Also, it is an indication that it contained high fibre that supported the growth of bacterial species involved in acetate production (France and Dijkstra, 2005). The legumes' propionate and butyrate concentrations were far below the recommended amount. This supports the findings that most woody legumes are used as a protein supplement and, in most cases, not as an energy supplement as they tend to contain high CP contents (Topps, 1992; Dzowela *et al.*, 1995).

The higher branched chain VFAs (valeric and isovaleric acids) concentrations of GS was a result of its ammonia concentration. This is because, during substrate fermentation in the rumen, amino acid deamination and further breakdown of the amino acid carbon skeleton led to the production of NH_3-N and branched chain VFAs respectively (France and Dijkstra, 2005). The branched chain VFAs concentration (butyric, isobutyric, valeric and isovaleric acids) tended to be positively related to NH_3-N degradation.

The forage controls (i.e. LP and TA) have shown that they can be used as positive and negative controls. The chemical content of LP falls within that of a good quality forage (i.e. high CP and EE, and low fibre and lignin content) while that of TA falls within that of low-quality forage (low CP and EE, and high fibre and lignin content) (Ball *et al.*, 2001; Newman *et al.*, 2006; Van Saun, 2006). The forage controls were different in nutrient utilisation. LP was of higher nutrient degradability, higher ammonia content, higher volatile fatty acids, and lower methane content than TA. The nutrient degradability of LP was more than 650 g / kg which was within the expected range of 612 – 696 (Chapman, 1985) for high-quality forages, while that of TA

was less than 550 g /kg (Leng, 1990) as expected for low-quality forages. The higher and lower nutrient degradability values recorded in LP and TA respectively were a reflection of their variable CP and fibre contents. It has been established that high CP enhances microbial growth and multiplication in the rumen, which in turn facilitates the fermentation rates, thus improving substrate degradability (Van Soest, 1994; Al-Soqeer, 2008; Njidda and Nasiru, 2010); while higher fibre content suppresses the activities of the rumen microbes thus leading to a reduction in the rate of carbohydrate fermentation (Wilson and Hatfield, 1997).

The rumen fluids obtained from different sheep gave little or no difference in the molar proportion of individual or total VFAs, pH, and ammonia concentration. This indicates that an almost similar mix of microflora was present in the rumen fluids which were involved in the degradation of the substrates (this was investigated further in Chapter 3). This supports the findings of Mabjeesh *et al.* (2000) and Goldman *et al.* (1987), where sheep rumen fluids or sources of inoculum had negligible effect on fermentation parameters.

The strong negative correlation of CP and fibre parameters (NDF and ADF) were in agreement with those of Abdulrazak *et al.* (2000), while the very weak positive correlation (non-significant) between CP with ADL was not in agreement with those of Abdulrazak *et al.* (2000). This might be that some of the forages, especially the legumes, contained high ADL with high CP, and the CP was supporting the growth of microbes. Woody legumes are known with high CP coupled with higher lignin content (Van Saun, 2006). The strong positive correlations of CP with metabolites (TP, TT, and CT) were not in agreement with the findings of Abdulrazak *et al.* (2000), Ndlovu and Nherera (1997) and Frutos *et al.* (2002). This can be interpreted to mean that some forages with high CP content contained higher polyphenolics, which was the case with the woody legumes (Garcia *et al.*, 1996; Aye and Adegun, 2013). The strong positive correlation that existed between each of the polyphenolic compounds was in agreement with the findings of Abdulrazak *et al.* (2000) and Frutos *et al.* (2002). The strong negative correlations of polyphenolic compounds and fibre fractions were in agreement with the findings of Khazaal and Ørskov (1994) and Tolera *et al.* (1997), but were not in agreement with those of Abdulrazak *et al.* (2000), Reed (1986) and Nsahlai *et al.* (1994), while the very weak positive correlation of PPCs with ADL was in agreement with those of Nsahlai *et al.* (1994) and Abdulrazak *et al.* (2000). The polyphenolic compounds negative correlations with hemicellulose were not in agreement with those of Abdulrazak *et al.* (2000). The differences in results can be attributed to the forage types, especially the woody legumes that produce high doses of polyphenolics; polyphenolic compounds are expected to reduce substrate (i.e. cellulose, pectin, hemicellulose, soluble sugars and starch) fermentation, which is what was

recorded in this study. In agreement with the existing findings (Reed, 1986), fibre fractions were strongly correlated with hemicellulose and cellulose and also with each other. ADL positive correlations with hemicellulose and cellulose were in agreement with those of Reed (1986), while the very weak negative correlations with NDF and ADF were not in agreement with the findings of Reed (1986). This might be attributed to forage type, as some forage types, such as legumes, do have higher lignin but contain lesser fibre components (Bamualim *et al.*, 1980; Broderick, 1995).

The positive correlation of CP with gas production, and gas production constants (rate of gas production and potential of maximum gas yield), were in agreement with the existing literature (Nsahlai *et al.*, 1994; Ndlovu and Nherera, 1997; Larbi *et al.*, 1998), but not in agreement with the findings of Getachew *et al.* (2004) where the CP was negatively correlated to gas production at 6h, 24h and 48h respectively. The negative correlation that exists between CP and lagphase (i.e. the hours required to produce the maximum gas) indicates that higher CP content in forages leads to lesser time for microbial degradation of organic matter content. The strong positive correlation that exists between CP and IVOMD (12h) supports the existing literature (Cherney and Marten, 1982; Russell, 1986; Getachew *et al.*, 2004). The positive correlation of CP with ammonia was consistent with findings of Getachew *et al.* (2004), and this indicates that CP degradability was high in all forages. The negative correlations of cell wall components (i.e NDF, ADF, and ADL) with gas production and gas production constants were consistent with existing literatures (Nsahlai *et al.*, 1994; Ndlovu and Nherera, 1997; Larbi *et al.*, 1998; Abdulrazak *et al.*, 2000; Getachew *et al.*, 2004). The strong positive correlation that exists between the cell wall components and the lagphase, shows that a high amount of cell wall contents in a forage makes microbial degradation of such forage in the rumen to be longer. This supports the findings that fibrous feed requires longer retention time in the rumen for effective degradation to take place (Lechner-Doll *et al.*, 1991).The strong negative correlation that existed between fibre fractions and IVOMD at 12h reduced to a non-significant correlation with further incubation time. This can be interpreted to mean that much microbial degradation had not taken place at 12h, which made the fibre content have a very high negative effect on IVOMD. However, with further incubation time, when microbial degradation has started, the fibre content exhibited a less negative effect on the IVOMD. The negative correlations that exist between fibre content and ammonia support the existing literature (Cherney and Marten, 1982; Russell, 1986; Larbi *et al.*, 1998; Getachew *et al.*, 2004); though the negative correlation of fibre with ammonia was increasing with increase in incubation time. This can be interpreted to mean that an increase in incubation led to the higher availability of ammonia and less amount

of fibre . This shows that microbial degradation of the substrate took place with an increase in incubation time, and this is producing the ammonia needed for microbial growth and multiplication in the rumen for efficient fibre degradation (Van Soest, 1994; Al-Soqeer, 2008; Njidda and Nasiru, 2010). The poor positive correlations that were recorded between polyphenolic compounds and gas production at 12h were in agreement with the findings of Larbi *et al.* (1998), Khazaal and Ørskov (1994); Tolera *et al.* (1997), while the poor negative correlations that exist after 48h and 72h were consistent with the existing literature (Khazaal *et al.*, 1993; Abdulrazak *et al.*, 2000). This indicates that as at 12h, the polyphenolic compounds were not affecting the gas production and it might be that the forages have not yet been properly degraded, therefore not having much significant effect on the gas production. However, as the incubation time increased, the microbes have started to degrade the forages, therefore exposing them to the polyphenolic compounds which are known for their antimicrobial property (Patra and Saxena, 2009; Manasri *et al.*, 2012), and this has started to affect the gas production gradually. The poor negative correlation of polyphenolic compounds and gas production constants (i.e. rate of gas production, the potential of maximum gas yield, and lag phase) were consistent with the findings of Frutos *et al.* (2002). This supports the existing report that the polyphenolic compounds possesses an antimicrobial property, which tends to reduce the growth and/or activity of certain microbes that are involved in the fermentation process, therefore reducing the rate at which forage nutrients are degraded and affecting gas characteristics (Manasri *et al.*, 2012; Anantasook *et al.*, 2013). The polyphenolic compounds were poorly positively correlated to IVOMD at 12h, however with further incubation hours (i.e. 48h and 72h), a negative correlation was recorded which was consistent with existing literature (Khazaal and Ørskov, 1994; Frutos *et al.*, 2002). This indicates that the forages were not properly degraded at 12h of incubation but, as the incubation time elongates, microbes were degrading the forages and were exposed to the polyphenolic compounds which had a negative effect on them, therefore affecting the degradability. The poor negative correlations that exist between polyphenolic compounds (PPCs) and ammonia production at 12h of incubation indicate that a high contents of PPCs had a negative effect on the microbes that were degrading nitrogen from which ammonia was obtained. This supports the findings of Larbi *et al.* (1998), Rittner and Reed (1992) and Arthun *et al.* (1992), where polyphenolic compounds reduced nitrogen degradability in the rumen and availability to livestock. The negative correlation that exists between the lagphase with the rate of maximum gas production and potential of gas yield, demonstrates that forages with a faster rate of degradation yield more gas at a shorter retention time in the rumen (Menke *et al.*, 1979; Cone *et al.*, 1996). The very strong positive correlation that existed between the rate and potential of gas yield (i.e. extent) supports the findings of

Cone *et al.* (1996), Cone and Becker (2012) and Menke *et al.* (1979), where the forage with the highest rate of gas production recorded the highest extent of gas production. Total gas production at 12h, 48h and 72h were strongly positively correlated to the rate and potential of gas yield (i.e. extent), indicating that highly fermented forage will produce more gas and reach the maximum yield of gas production at a faster rate (Cone and Becker, 2012). Total GP at 12h, 48h and 72h were negatively correlated to lagphase; this demonstrates that forages producing high total gas production will not take longer time in the rumen for the gas to be produced. The strong positive correlations that existed between IVOMD at 12h, 48h and 72h with total gas production at 12h, 48h and 72h respectively, and gas production constants (i.e the rate and potential of gas production), were not in agreement with the findings of Getachew *et al.* (2004), where a very poor relationship and negative correlations were recorded between in vitro true digestibility with gas production and gas production constants, but were in agreement with the findings of Apori *et al.* (1998). The results obtained in this study support the existing theory that higher gas production indicates that higher organic matter has been degraded (Menke *et al.*, 1979; Cone and Becker, 2012). The negative correlations between IVOMD at 12h, 48h and 72h and lagphase show that forage where organic matter is highly degraded required lesser retention time in the rumen (Cone *et al.*, 1996). The positive correlations between ammonia at 12h, 48h, and 72h and gas production constants (i.e. rate of gas production and potential of gas yield), total gas production at 12h, 48h and 72h, and IVOMD at 12h, 48h and 72h respectively were not in agreement with the findings of Getachew *et al.* (2004), where negative relationships were recorded. The obtained result in this study was in support of the existing findings that higher degradation of organic matter in the rumen is a result of the increase in the synthesis of microbial protein, i.e. a source of ammonia (Cone and Becker, 2012), as fermentation is measured using gas production and its constant (Menke *et al.*, 1979). The negative correlation between ammonia and lag phase indicates that the forages that produce more ammonia in the rumen require less time for their degradation or fermentation. The positive correlation between methane content at 12h and gas production constant, total gas production at 12h, and IVOMD at 12h, is an indication that higher organic matter degradation in the rumen leads to the production of more gas, and also leads to the generation of more methane.

A strong positive correlation existed between tVFAs at 12h, 48h and 72h and gas production constants, total gas production, at 12h, 48h and 72h, ammonia at 12h, 48h and 72h, IVOMD at 12h, 48h, and 72h and methane at 12h, and 48h. This supports the findings of Blümmel *et al.* (1997), Getachew *et al.* (2004), Getachew *et al.* (2002) and Paul and Beauchamp (1989), where a strong relationship was recorded between VFAs and total gas production, in vitro true

digestibility and ammonia-nitrogen respectively. The obtained correlation was not in agreement with the findings of Getachew *et al.* (2004), where tVFA was poorly negatively correlated with the ammonia-nitrogen. The strong correlation tVFAs had with ammonia concentration in this study might be the reason why it was strongly correlated to methane content, as a positive relationship was found between ammonia production and methane production at lower pH when cattle were fed forage, i.e timothy hay with concentrate (Lana *et al.*, 1998). Also, the strong correlation between tVFAs and total gas production supports the existing information that gas production is a reflection of the amount of VFAs produced from a degraded forage (Getachew *et al.*, 2004). The negative relationship that exists between each molar proportion of individual and total volatile fatty acids and lag phase may be interpreted to mean that the more energy is released, the lesser the time the feed is being retained in the rumen, as the nutrients would have been exhausted over a shorter time (Cone *et al.*, 1996; Cone and Becker, 2012). The strong positive correlation that exists between some molar fractions of tVFAs (i.e. acetate, propionate and butyrate) and total gas production at each of the incubation times, especially at 12h, was in agreement with the findings of Getachew *et al.* (2004). The strong positive correlation that exists between valerate, especially at the later incubation hours (48h and 72h), and total gas production was not in agreement with the findings of Getachew *et al.* (2004). The discrepancies in results might be due to the forage amino acid composition and deamination composition of the forages (Hume, 1970a), although not estimated in this study. Also, branched-chain VFA concentration has been found to be positively related to ammonia degradation and, from this study, the amount of ammonia produced tends to increase with an increase in incubation time. This might have been the reason why a strong correlation was recorded between valerate and total gas production at later incubation times.

The positive correlation that exists between some molar fractions of tVFAs (i.e. acetate, propionate and butyrate) and ammonia, especially at 12h, was not in agreement with the findings of Getachew *et al.* (2004) and (Hume, 1970a), but the positive correlation of the branched chain VFA (i.e. valerate) and ammonia was in agreement with findings of Getachew *et al.* (2004). The discrepancies might be based on the forage used, as some forages contain antinutritive substances that inhibit the growth of microbes involved in the degradation of nutrients for the release of VFAs and ammonia. For a good quality forage with higher ammonia content, a higher VFAs is expected. Rumen microbes require nitrogenous compounds, such as ammonia, to facilitate their growth and activity on a substrate (Satter and Slyter, 1974), thus leading to higher substrate degradation from which VFAs are obtained.

The positive correlation that existed between molar fractions of tVFAs and IVOMD, especially at 12h, for most fractions except valerate was in agreement with the findings of Getachew *et al.* (2004). This can be interpreted to mean that forages that have their organic matter highly fermented produce higher molar fractions of tVFAs. A positive relationship exists between the molar fractions of tVFAs and methane, especially at 12h, except for valerate. Acetate and butyrate do have a strong relationship with methane (Kamalak *et al.*, 2002), while propionate produces an inverse relationship with methane (Wolin, 1960). However, the lower positive correlation of acetate with methane and higher positive correlation of propionate with methane in this study might be that the obtained carbon skeleton from amino acid deamination is producing a different form of VFA (Allison, 1970). For instance, glycine fermentation can lead to the production of acetate and ammonia without CO_2 release.

2.8 Conclusions

The Nigerian grasses are of low nutritive quality due to various different factors, i.e. low soluble fraction (CP and EE) and high structural carbohydrates (NDF, ADF and ADL), and low nutrient utilisation in terms of low nutrient degradability, low ammonia and tVFAs (i.e. sources of protein and energy) and high methane contents. This indicates that they are not capable of meeting the nutrient requirements of animals. Although *A. gayanus* seems to be exceptional, in that it has better degradability than other tropical grasses, it still has a significant portion of its energy being wasted in the form of methane; therefore it is classified as a low-quality forage. In general, concerning the nutrient fermentation profiles apart from methane content, the grasses can be grouped into 2 categories: 1) low-quality (AG and PP), and 2) very low-quality (BD and PM). However, with respect to methane content they can be grouped as follows: 1) low-quality (PP and PM), and very low quality (AG and BD). These grasses seem similar to the negative control (i.e. *T. aestivum*) in their chemical composition and *in vitro* fermentation properties. They have also shown that they were of little nutritive value and were unable to meet the nutrient requirements of animals for growth and production.

The legumes can be used as protein supplements, due to their high CP and ammonia contents, and not as an energy source due to low tVFAs. However, *L. leucocephala* is more preferred than *G. sepium* because it has its protein protected from ruminal microbes, which modulated the characteristics of rumen fermentation and led to a reduction in methane production, and it had higher TAC which may aid in boosting the immune system of an animal.

The British forages appeared to be of variable quality, where LP can be regarded as a better quality forage than TA. Clearly LP contained higher soluble fractions, lower structural carbohydrates, and higher degradability than TA. Therefore, LP and TA can be used as positive and negative controls, respectively, for the Nigerian grasses supplemented with or without the legumes.

Indeed, the observed changes in degradability and fermentation in this study were considered as indicators of changes in rumen microbial activity. Therefore, it may help to examine the rumen microbial composition to understand the impact of different forages, especially grasses, on various microbial populations. For this purpose, the studies were extended to investigate the influences of these forages on rumen microbial composition (see Chapter 3). The results of this microbial examination (Chapter 3), alongside the results of this chapter, were then used to select a subset of the most appropriate forages for possible improvement by applying fungal treatments in chapters 5 and 7.

Chapter 3.

Changes in Rumen Microbial Populations during the *in vitro* degradability of different forages

3.1 Introduction

As stated in previous chapters, tropical forages during the dry season are usually of low quality (Preston, 1982; Leng, 1990). Thus, they require further improvement and/or supplementation before they are capable of supporting adequate animal growth and production (Pérez *et al.*, 2002; Krause *et al.*, 2003; Franzel *et al.*, 2014; Shirkavand *et al.*, 2016). In the quest to improve such low-quality forages, there is the need to evaluate their nutritive quality to assess their feeding potential before adopting a suitable improvement strategy. Forage nutritive evaluation can be achieved by determining the chemical composition, nutrient digestibility or degradability, and microbial composition during rumen fermentation. In most cases, the chemical constituents (CP, NDF, ADF, and ADL etc.) of a forage can be used to predict digestibility or degradability and vice versa (Weiss *et al.*, 1992; Noblet and Perez, 1993; Khazaal *et al.*, 1995; Lopes *et al.*, 2015a; Lopes *et al.*, 2015b; Sol *et al.*, 2017; White *et al.*, 2017). However, these methods cannot be used to predict the rumen microbial biomass or composition (i.e. bacteria, archaea, protozoa and fungi) that are responsible for nutrient degradation. This is because, even when animals are fed with the same forage, the microbes can vary due to animal factors or conditions (Martínez *et al.*, 2010), changes in rumen pH (Russell and Wilson, 1996), forage factors i.e. use of different parts, stage of growth and size (Pérez *et al.*, 2002), and adaptation time (Lengowski *et al.*, 2016). Also, the *in vitro* gas production (i.e. a measure of degradability) of straws has been found to be inversely related to microbial biomass (Blümmel *et al.*, 1997). This is one of the reasons why measurements of *in vitro* gas production do not correlate consistently with the results of animal trials (Liu *et al.*, 2002), and that is why the influence of such forages on the microbial composition needs to be evaluated, to complete the whole process of nutritive evaluation.

In a related study (see chapter 2), the chemical composition and *in vitro* degradability of four kinds of Nigerian grasses (PP, PM, AG, and BD), two legumes (LL and GS) and forage controls (LP, and TA) were determined. The study was able to aid in the selection of two Nigerian grasses that may be best suited for further improvement, with or without a selected legume as a supplement, alongside UK forages as controls or a benchmark. Nevertheless, it may be more helpful if the possible changes in rumen microbial composition were also evaluated to help identify forages that are supporting microbial multiplication, which is a basic pre-requisite

needed in forages that can be biologically improved. Therefore, the studies of chapter 2 were extended to examine the influence of each forage type on the microbial composition of rumen fluid to aid in the final selection of the two most suitable Nigerian grasses, with or without a legume, that will be improved in further studies. This extended study was conducted by using the buffered rumen fluid (RF) collected before and after incubation for 48h with different forage types in the *in vitro* degradability study (chapter 2).

Determination of microbial composition can be carried out using several techniques, such as cultivation (Chen *et al.*, 2007) and microscopy (Dehority and Odenyo, 2003; Bootten *et al.*, 2011) based techniques. In this study, the microscopy approach was used due to its usefulness in getting a more accurate microbial cell count than the cultivation technique (Skinner *et al.*, 1952; Hobbie and Fletcher, 1988; Riis *et al.*, 1998; Lawlor *et al.*, 2000). However, to obtain the best count, the macromolecules (i.e. protein and nucleic acids) of cells need to be intact and un-affected (Alberts *et al.*, 2002; De Araújo and Roussos, 2002). For this purpose the use of fixatives such as mercurial compounds and picrates (Fox *et al.*, 1985; Srinivasan *et al.*, 2002; Nowacek and Kiernan, 2010), oxidizing agents (i.e. potassium permanganate, potassium dichromate and osmium tetroxide), alcohols (i.e. methanol and ethanol) and aldehydes (i.e. formaldehydes and glutaraldehydes) (Fox *et al.*, 1985) have been considered. Paraformaldehyde (PFA) was applied in this study because of its usefulness in cross-linking protein without destroying protein structure (Hopwood, 1985; Nowacek and Kiernan, 2010). Also, the cells or tissues need to be stained with dyes or fluorochrome to facilitate better observation of the fixed cells by microscope (Winsor, 1994; Howat and Wilson, 2014). This chapter therefore presents data on the influences of each forage type on the microbial composition (i.e. bacterial and fungal count) as determined by observing the fixed and stained rumen fluids under a microscope. The RF samples were obtained before and after incubating the same four Nigerian grasses (i.e. *P. maximum*, *P. purpureum*, *A. gayanus*, and *B. decumbens*), two legumes (*L. leucocephala* and *G. sepium*) and two forage controls (*T. aestivum, negative* and *L. perenne, positive*) with RF for 48 hours (chapter 2).

The study was divided into three sub-experiments. In experiment 1, two fixative concentrations and two staining agents were tested for their suitability to facilitate better bacterial counting in the sheep RF that were used for the *in vitro* experiment in chapter 2. In experiment 2, total fungal sporangia in each sheep RF used for the *in vitro* study were counted after fixing with 4% PFA solution. In experiment 3, total bacterial cells and fungal sporangia were counted in the un-incubated RF (i.e. blank) as well as different RF following incubations with each of the above listed forages for 48h, as described in chapter 2 and section 3.2 of this chapter.

3.1.1 Objectives

To determine:

1. The bacterial counts in the un-incubated RF before its use for the *in vitro* study and in the RF obtained before and after its incubation with each of the forage types (i.e. Nigerian grasses, legumes and forage controls) separately for 48h.
2. To determine the fungal count in the un-incubated RF before its use for the *in vitro* study and in the RF obtained before and after its incubation with each of the forage types (i.e. Nigerian grasses, legumes and forage control) separately for 48h as described in chapter 2.

3.2 Materials and Methods

3.2.1 *Preparation of un-incubated Rumen fluid for microbial studies*

The rumen contents collected for the *in vitro* studies (chapter 2, section 2.3.3.4) from four sheep were strained to obtain RF, the RF collected were then pooled to make RF from 2 sheep, as described in chapter 2 (section 2.3.3.1). However, in this chapter, the four RF were prepared in different ways to suit various microbial tests as described below. The RF of each sheep was poured into a 1 litre sterilized brown glass-stoppered bottle and mixed thoroughly by manual shaking. The mixed RF was then distributed into a series of sterile polypropylene (50ml) bottles. The bottles were screw capped and kept under anaerobic conditions until used. The samples were then kept on ice during transit to minimize potential changes in the microbial population. On arrival in the laboratory, each bottle containing RF was flushed with CO_2, screw capped and stored at -20^0C for three weeks. Each frozen RF was then processed as described below before use in the three subsequent experiments.

3.2.1.1 *Thawing and fixing of RF for microbial studies*

The frozen RF of each sheep was thawed at 39^0C in an incubator (IKA KS 4000 I control) for 12h, which was reported (Chaudhry and Mohamed, 2012) to be needed for the revival of rumen microbes from freezing. After adequate mixing of each thawed RF, about one ml of RF was fixed in nine ml of either 2% or 4% PFA solution (prepared in sterilised distilled water) for bacterial counting. For fungal counting, one ml of the thawed RF was directly fixed in 4% PFA. The fixed samples were then stored at 4^0C for 24h to complete fixing of microbial cells before counting (Hopwood, 1985; Srinivasan *et al.*, 2002; Howat and Wilson, 2014) under an epifluorescence microscope (Nikon Eclipse Ci, CoolLED pE-300-W, Japan).

3.2.1.2 *Experiment 1: Comparing different fixatives and staining agents for counting total bacterial cells (TBC) in different thawed RF from sheep*

Two different fluorochromes, i.e. acridine orange and SYBR gold as described by Federation and American Public Health (2005) and Tuma *et al.* (1999) respectively, and two different fixatives (i.e. 2% and 4% PFA) were compared for their suitability to study total bacterial counts in different samples of thawed RF. Several dilutions (10^1 to 10^9) were prepared in Phosphate buffer saline solution (2x, pH 7.3; see appendix 3.1) to determine the optimum dilution to obtain the best bacterial count. One ml of the fixed sample from each of the two fixative concentrations was used, with the two above-mentioned fluorochromes respectively at different dilutions, to ascertain the most suitable dilution factor, fixative, staining agent and their combination for

microbial counting in different samples of thawed RF of different sheep (detailed procedures are presented in **Appendix 3.1**).

3.2.1.3 *Experiment 2: Counting of total fungal cells (TFC) in thawed RF from different sheep*

The fungal sporangia counts were carried out using calcoflour white as a staining agent (Harrington and Hageage, 2003) on RF fixed in 4% paraformaldehyde (this was identified as a more suitable fixative in experiment 1). Several dilutions (10^1 to 10^5) in Phosphate buffer saline solution (1X, pH 7.3; **see Appendix 3.2**) of each fixed RF were compared for their suitability to obtain the best fungal sporangial count (detailed procedures are presented in **Appendix 3.2**).

3.2.2 *Experiment 3. Microbial counting in RF after incubation with forages for 48 hours*

This involves the preparation of the RF obtained from the *in vitro* incubations with forage samples for 48h in anticipation of microbial (i.e. bacterial and fungal) counting as described below.

3.2.2.1 *Preparation of RF from in vitro incubations with forage samples for 48h for microbial counting*

As described in chapter 2, the rumen contents of each slaughtered sheep were strained through 4 layers of cheesecloth to obtain RF that was mixed with the buffer to prepare the buffered inoculum. The buffered inoculum was eventually used to incubate forage samples (n=8) in 50 ml centrifuge tubes for three different times (12, 48, 72 h) in duplicate. After each incubation, the tube with its contents was centrifuged to obtain supernatants of RF and washed residues (chapter 2). For microbial counting, only the supernatants of RF after 48 hours of incubation were flushed with CO_2 and stored at -20^0C for around a month before their use for microbial counting. The RF from forages incubated for 48h were chosen, because low quality forages show an appreciable degradation at longer retention time in the rumen, and at this point optimal microbial counts can be recorded. Immediately before counting, the tubes were removed from the freezer for thawing at 39^0C in an incubator for 12h. The replicated thawed samples of RF for each forage were then pooled into a sterile 100 ml polypropylene bottle and mixed, before fixing 1ml of each thawed RF in 9ml of 4% PFA and staining as described above and below.

3.2.2.2 *Experiment 3: Counting of bacterial and fungal cells in thawed RF after in vitro incubations with forages for 48h*

The fixed RF were stained with either SYBR gold (identified in experiment 1 as the best staining agent) for microbial counting or lactophenol blue for fungal sporangial counting. The prepared samples were then examined under the microscope using a magnification of 100x (i.e. for bacterial cell counting) or 20x (i.e. for fungal sporangia counting), with a 10x eyepiece. The detailed procedures were the same as described in section 3.2.1.2. The obtained total bacterial and fungal counts were corrected by using the counts in the blanks (i.e. un-incubated RF without forages).

3.2.3 *Statistical analysis*

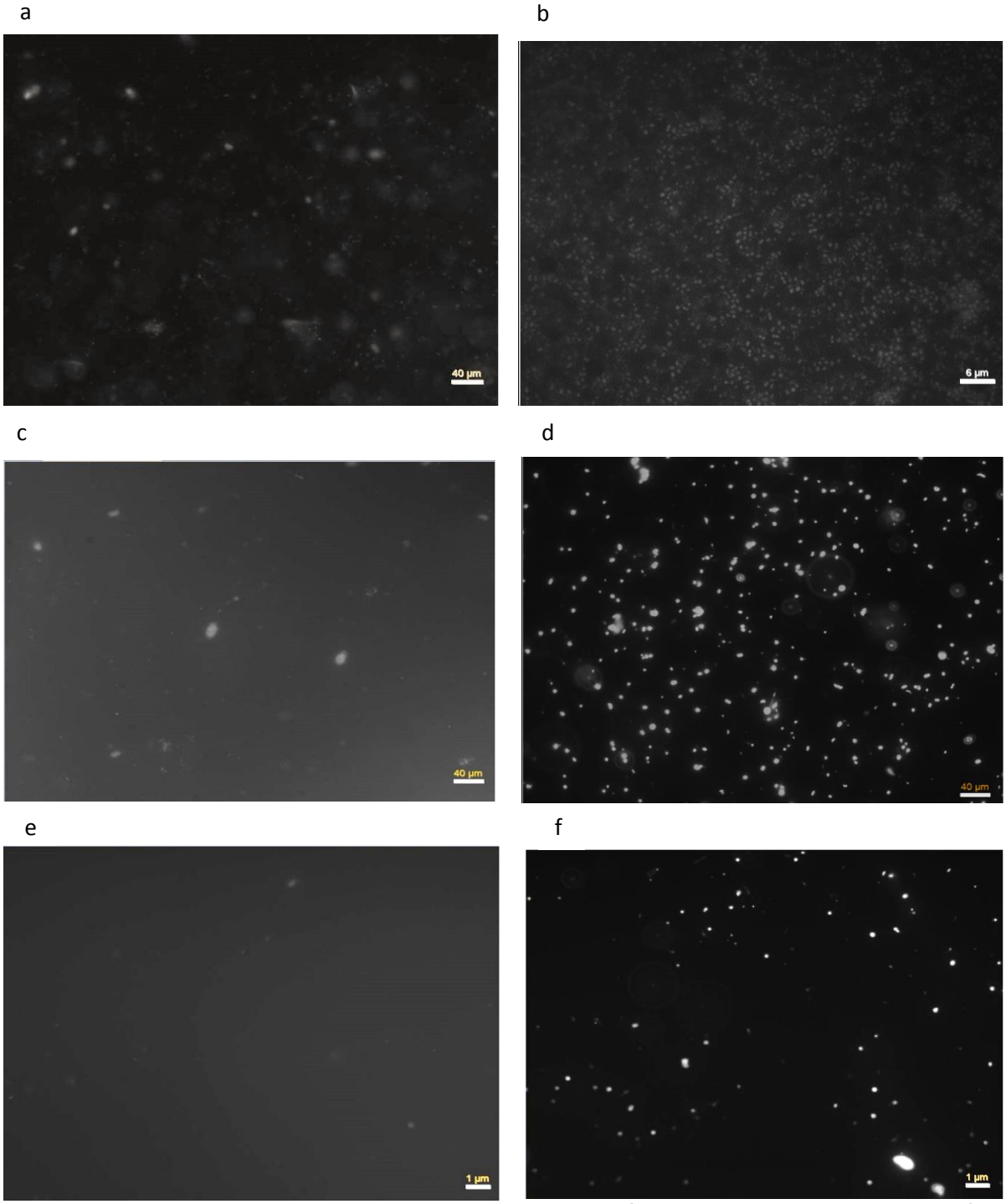
The data for the bacterial and fungal counts were analysed by using GLM in Minitab 16 software to determine either the main effects of sheep and fixative alongside their interaction (experiment 1), or sheep effect on TFC only (experiment 2), or only the forage effect on both TBC and TFC after 48h of incubation (experiment 3). Visual illustrations of various microbial and fungal cells were also provided by including the magnified images of the microscopic examinations of different RF as pictures.

3.3 Results

3.3.1 *Visual illustrations for the effects of sheep RF, fixative, staining agents and in vitro degraded forages on TBC and TFC*

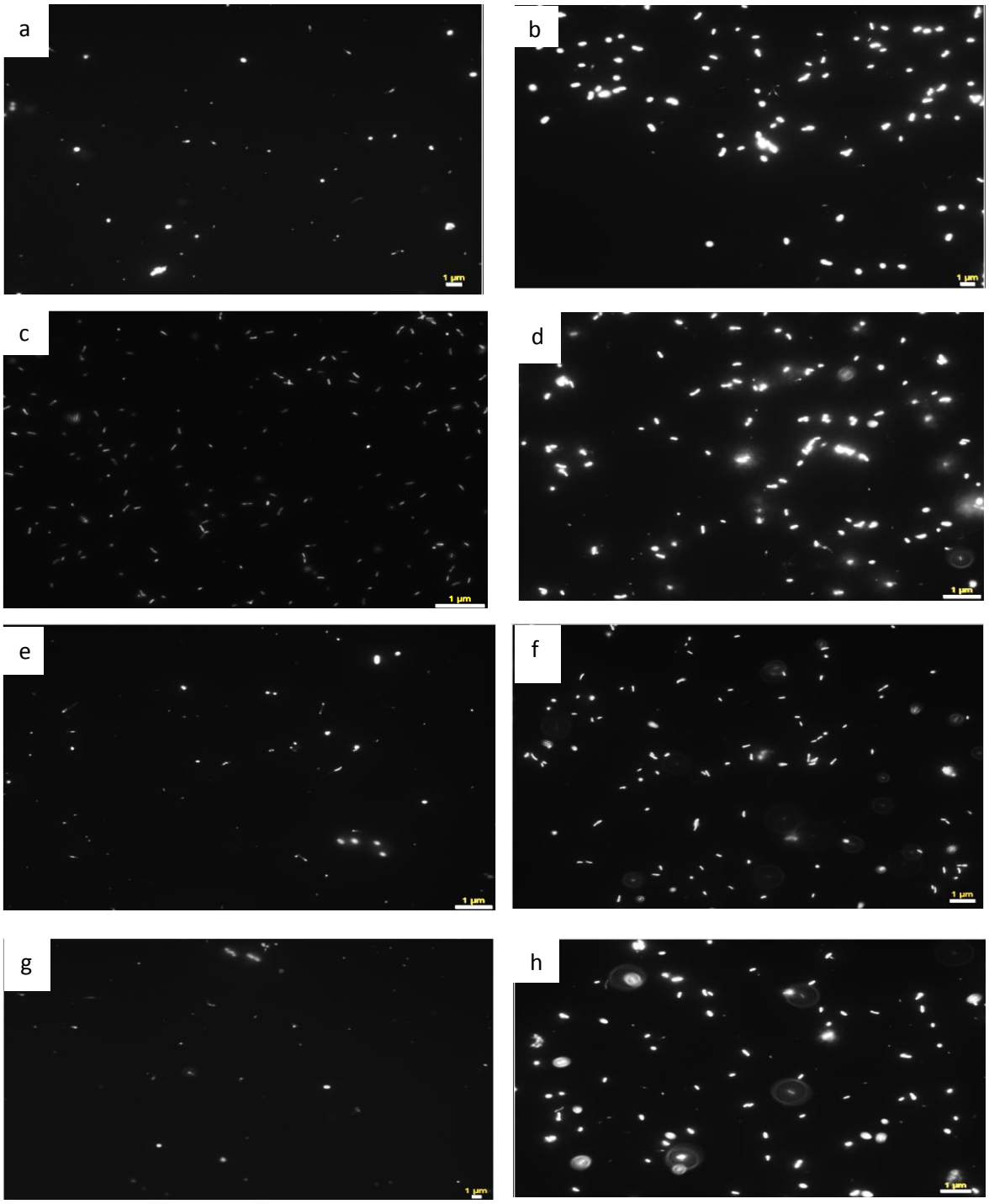
Pictures 1 and 2 below show respectively the effects of fixative and stains on the total bacterial count in RF of sheep. Picture 3 shows the TBC from each of the rumen fluid of the sheep when stained with the best stain. Picture 4 shows the TFC from each of the rumen fluid of the 4 sheep when fixed with 4% PFA. Picture 5 and Picture 6 show the TBC and TFC respectively of the buffered inocula from *in vitro* degraded forages when fixed with 4% PFA. Statistical analysis was not performed for the various stains, as numerical values were difficult to obtain when acridine orange was used as the stain.

Picture 1 illustrates the images of two types of fluorochrome (Acridine orange and SYBR Gold) that were investigated for their staining capability of the bacterial cells. SYBR gold was found to be more effective for the staining with a brighter view than Acridine orange. The following images helped in the selection of SYBR gold as the preferred stain for subsequent studies for microbial counting.



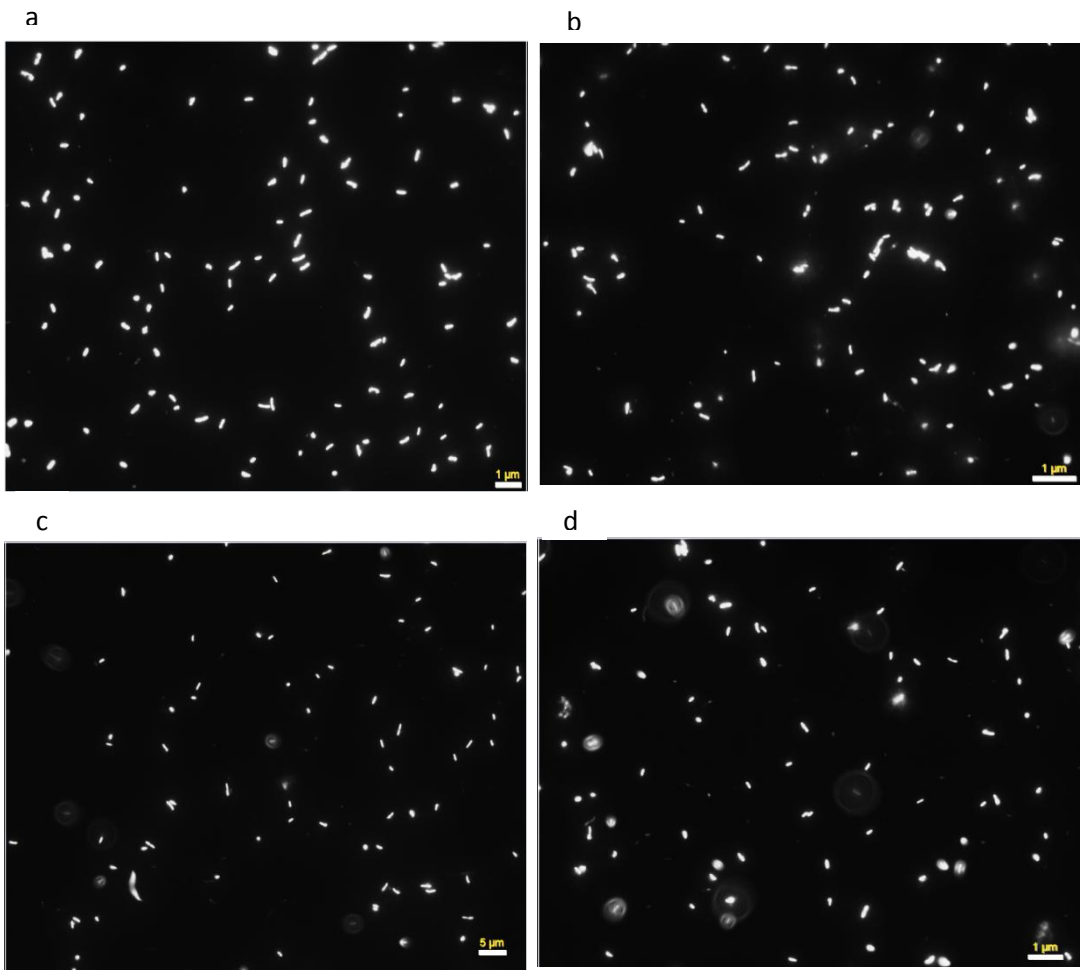
Picture 1. Rumen fluid stained with a) Acridine orange (10^2 dilution, 100X), b) Sybr gold (10^2 dilution, 100X), c) Acridine orange (10^4 dilution, 100X), d) Sybr gold (10^4 dilution, 100X), e) Acrdine orange (10^5 dilution, 100X), f) Sybr gold (10^5 dilution, 100X)

Picture 2 illustrates the images of microbial cells in RF from 4 sheep that were fixed in 2 different fixatives (2% PFA and 4% PFA). Based on these images, 4% PFA was identified to be the more suitable for maintaining the integrity of microbial cells.



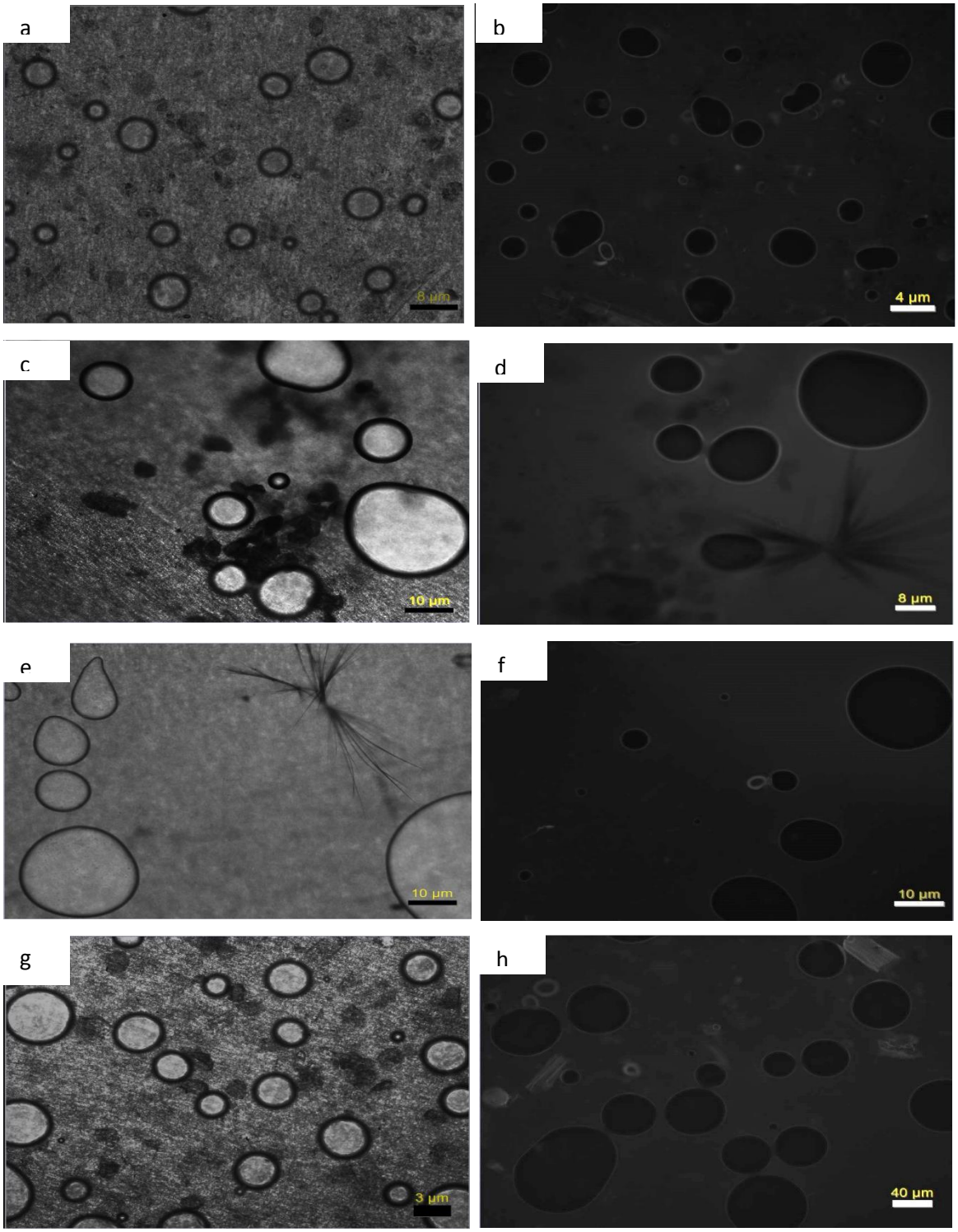
Picture 2. Rumen fluid fixed with a) Sheep 1, 2% PFA (100X), SYBR gold, b) Sheep 1, 4% PFA (100X), SYBR gold, c) Sheep 2, 2% PFA (100X), SYBR gold, d) Sheep 2, 4% PFA (100X), SYBR gold, e) Sheep 3, 2% PFA (100X), SYBR gold, f) Sheep 3, 4% PFA (100X), SYBR gold, g) Sheep 4, 2% PFA (100X), SYBR gold, h) Sheep 4, 4% PFA (100X), SYBR gold

Picture 3 illustrates the images of TBC in RF of different sheep with the best dilution factor (10^7). The TBC appeared to range from about 30 -300 cells/ field of view (FOV).



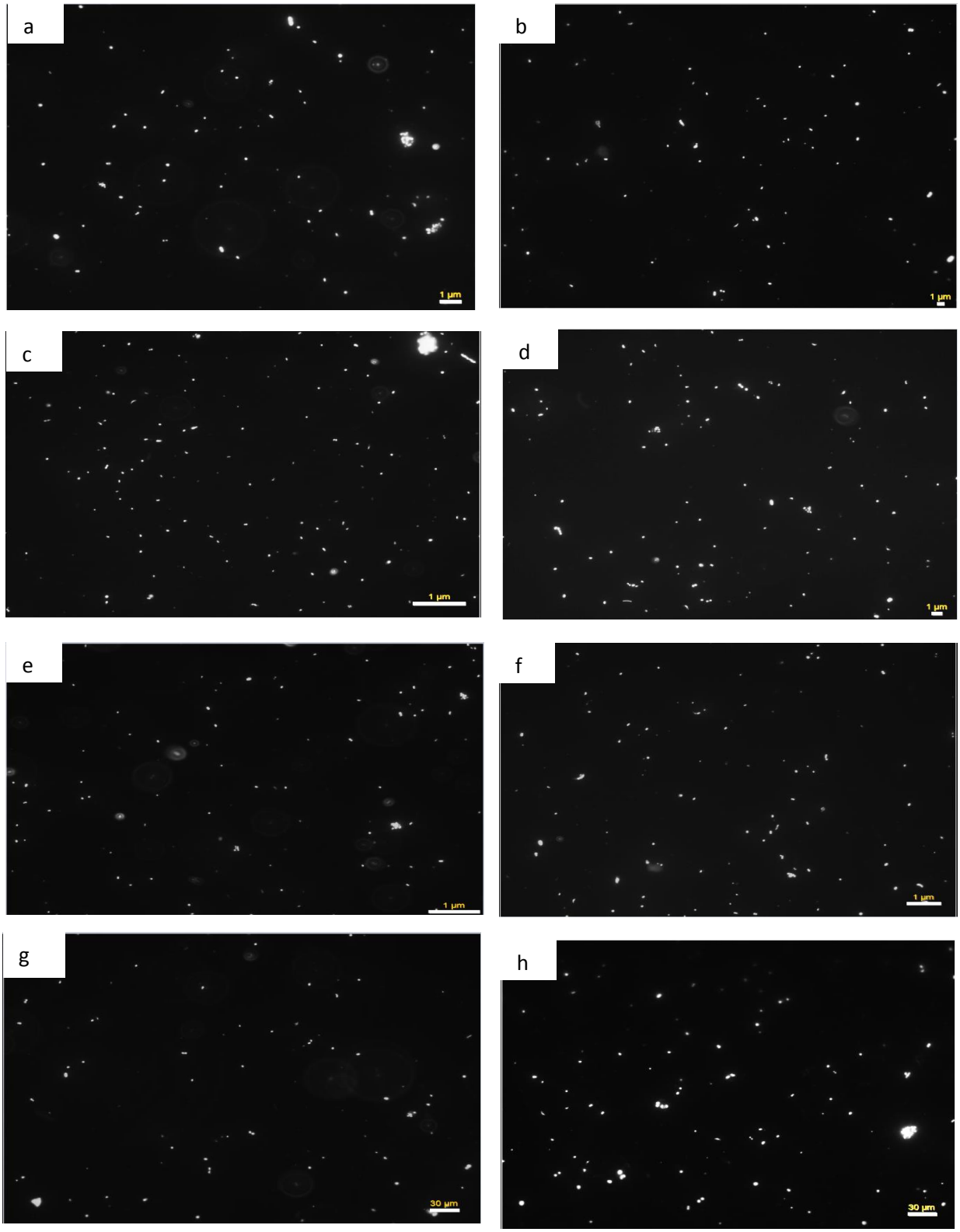
Picture 3. Total bacterial count images from a) Sheep 1, 4% PFA (100X), b) Sheep 2, 4% PFA (100X), c) Sheep 3, 4% PFA (100X), d) Sheep 4, 4% PFA (100X)

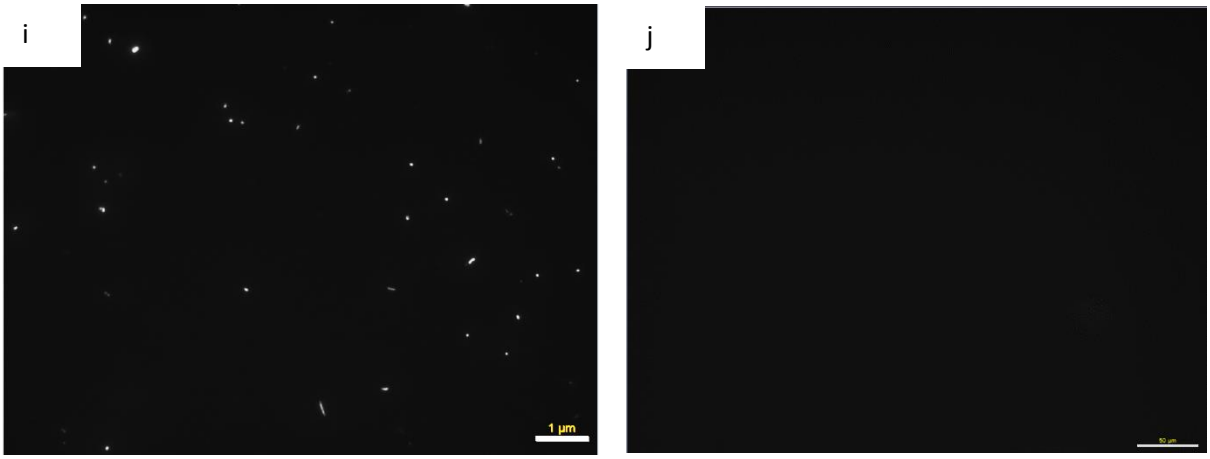
Picture 4 illustrates TFC from each sheep rumen fluid with the best dilution factor (10^2) from which about 1 - 50 cells / field of view (FOV) were obtained.



Picture 4. Total fungal count images from a) Sheep 1, 4% PFA (20X, ML), b) Sheep 1, 4% PFA (20X, UV light), c) Sheep 2, 4% PFA (20X, ML), d) Sheep 2, 4% PFA (20X, UV light), e) Sheep 3, 4% PFA (20X ML), f) Sheep 3, 4% PFA (20X, UV light), g) Sheep 4, 4% PFA (20X ML), h) Sheep 4, 4% PFA (20X, UV light). ML : microscope light UV: Ultraviolet light

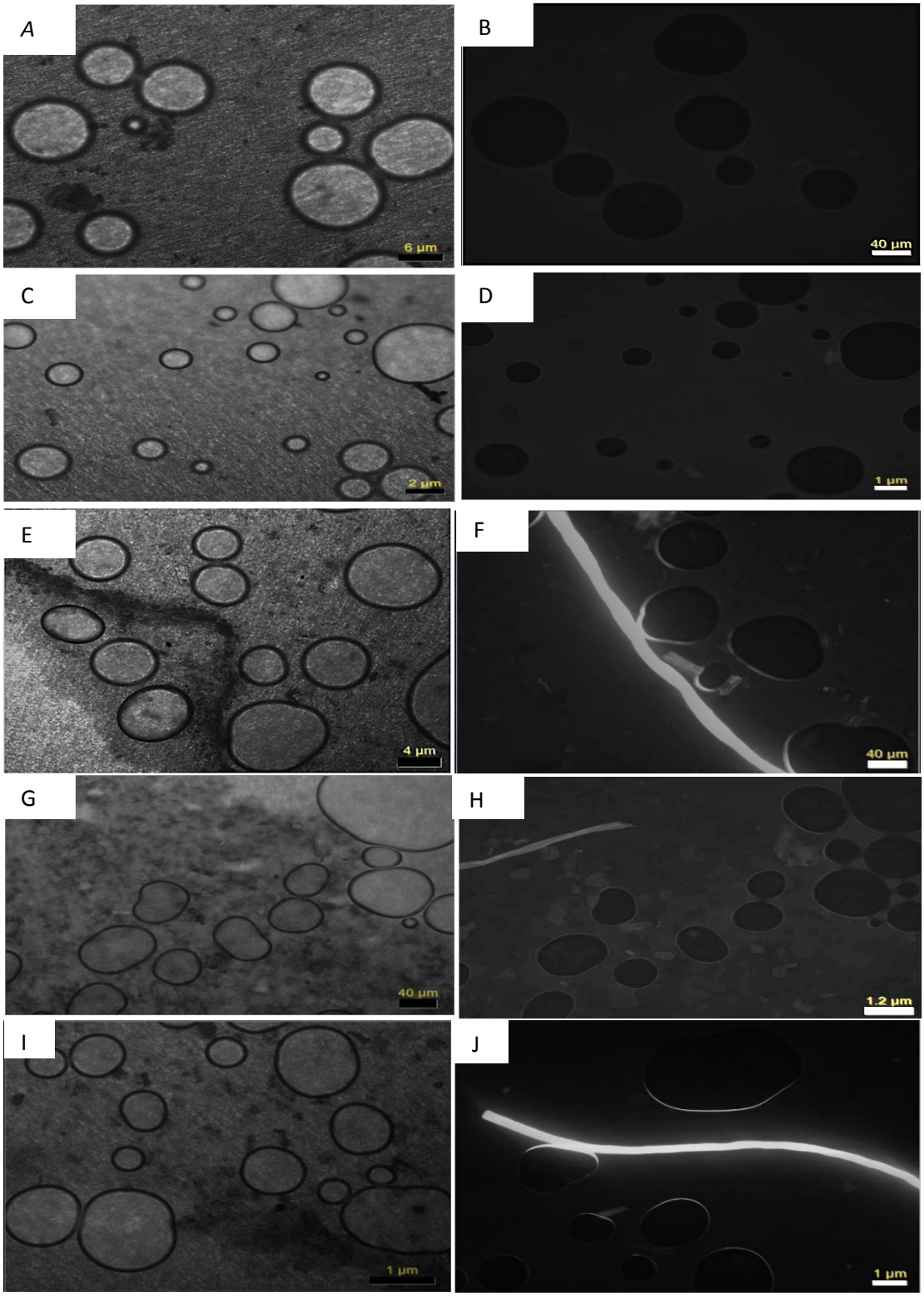
Picture 5 illustrates the TBC from the diluted (10^4) buffered inoculum following incubation *in vitro* with different forages in chapter 2. The studies showed around 30 -300 bacterial cells / FOV.

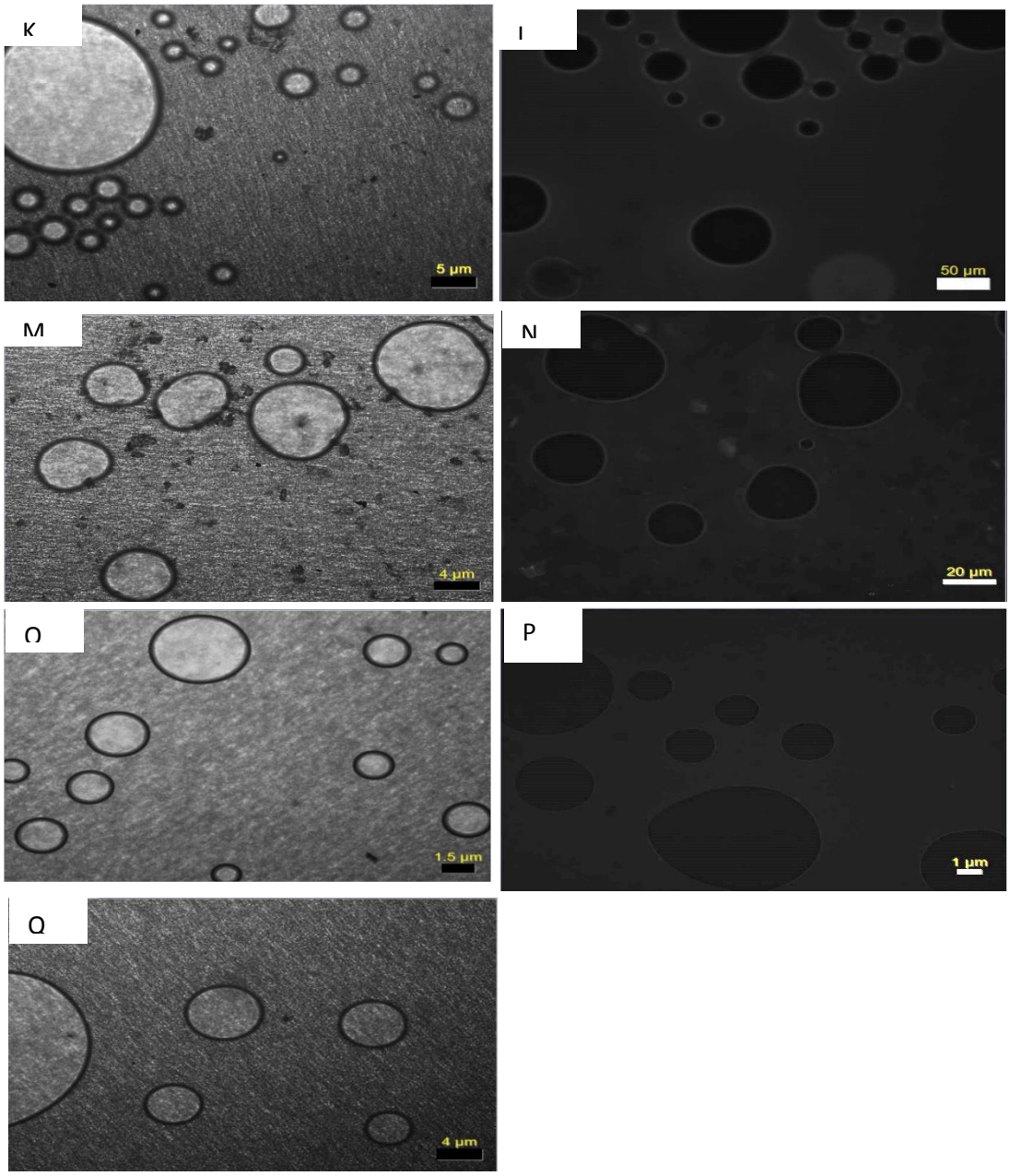




Picture 5. Total bacterial count images for a*) P. purpureum* (10^4 dilution), 4% PFA, 100X, b) *P. maximum* (10^4 dilution), 4% PFA, 100X, c) *B. decumbens* (10^4 dilution), 4% PFA, 100X, d) *A. gayanus* (10^4 dilution), 4% PFA, 100X, e) *G. sepium* (10^4 dilution), 4% PFA, 100X , f) *L. leucocephala* (10^4 dilution), 4% PFA, 100X, g) *L. perenne* (10^4 dilution), 4% PFA, 100X, h) *T. aestivum*((10^4 dilution), 4% PFA, 100X, i) Buffered inoculum without substrate (10^4 dilution) 4% PFA, 100X, j) Buffer alone (4% PFA, 100X).

Picture 6 illustrates TFC from the diluted (10^{1}) buffered inoculum after incubation *in vitro* with different forages. The studies suggested around 1- 50 cells / FOV (i.e. field of view).





Picture 6. Total fungal count images for A) *P. purpureum* (4% PFA, 20X, ML), B) *P. purpureum* (4% PFA, 20X, UVL), C) *P. maximum* (4% PFA, 20X, ML), D) *P. maximum* (4% PFA, 20X, UVL), E) *B. decumbens* (4% PFA, 20X, ML), F) *B. decumbens* (4% PFA, 20X, UVL), G) *A. gayanus* (4% PFA, 20X, ML), H) *A. gayanus* (4% PFA, 20X, UVL), I) *G. sepium* (4% PFA, 20X, ML), J) *G. sepium* (4% PFA, 20X, UVL), K) *L. leucocephala* (4% PFA, 20X, ML), L) *L. leucocephala* (4% PFA, 20X, UVL), M) *L. perenne* (4% PFA, 20X, ML), N) *L. perenne* (4% PFA, 20X, UVL), O) *T. aestivum* (4% PFA, 20X, ML), P) *T. aestivum* (4% PFA, 20X, UVL), Q) Buffered inoculum without substrate (10^1 dilution) 4% PFA, 100X.

3.3.2 *Experiment 1: The effects of sheep, fixative and their interaction on TBC / ml of thawed rumen fluid*

Table 26 shows that the main effects of sheep (RF) and the PFA concentration, as well as their interaction, recorded significance ($P < 0.05$) for the TBC (cells / ml). The RF influenced the TBC, with the RF from sheep two recording the highest TBC value (1.45 x 10^{12} cells / ml), followed by Sheep three and one (1.06 and 1.01 x 10^{12} cells / ml) and lastly by Sheep four (0.86 x 10^{12} cells / ml). Higher total bacterial counts (1.36 x 10^{12} cell / ml of RF) were observed when the sheep RFs were fixed in 4 % PFA than with 2% PFA (0.83 x 10^{12} cells/ ml of RF). The interaction between Sheep RF and PFA fixative concentration produced the highest TBC value (1.48 X 10^{12} cells/ml) when sheep two RF was fixed at 4% PFA, although the value was similar to the value (1.42 X 10^{12} cells/ml) recorded when fixed in 2% PFA. Sheep 4 RF when fixed in 2% PFA recorded the lowest TBC value (0.49 x 10^{12} cells / ml).

Table 26 Main and interactive effects of sheep rumen fluid and paraformaldehyde (PFA) fixative concentration on the TBC /ml of thawed rumen fluid

Item	TBC
Main effect of Sheep RF	
1 (10^{12} cells / ml)	1.01^{b}
2 (10^{12} cells / ml)	1.45^{a}
3 (10^{12} cells / ml)	1.06^{b}
4 (10^{12} cells / ml)	0.86^{c}
SEM (10^{12})	0.08
Main effect of Fixative concentration	
2% (10^{12} cells / ml)	0.83^{b}
4% (10^{12} cells / ml)	1.36^{a}
SEM (10^{12})	0.08
Interactive effect of Sheep RF and fixative concentration	
Sheep, 2% PFA	
1 (10^{12} cells / ml)	0.67^{c}
2 (10^{12} cells / ml)	1.42^{a}
3 (10^{12} cells / ml)	0.73^{c}
4 (10^{12} cells / ml)	0.49^{d}
Sheep, 4% PFA	
1 (10^{12} cells / ml)	1.36^{ab}
2 (10^{12} cells / ml)	1.48^{a}
3 (10^{12} cells / ml)	1.40^{ab}
4 (10^{12} cells / ml)	1.22^{b}
SEM (10^{12})	0.13

Means with different letters in the same column for the same effect were significantly ($P < 0.05$) different; SEM, standard error of mean.

3.3.3 *Experiment 2: The effect of sheep RF on the TFC / ml of thawed rumen fluid*

The sheep RF had a significant ($P < 0.05$) effect on the TFC (cells/ ml) as shown in Table 27. The highest TFC value (7.69 x 10^6 cell/ ml) was recorded in Sheep 1 RF, followed by Sheep 4 (6.53 x 10^6 cell / ml) and lastly by Sheep 2 and 3 RFs which were similar in TFC values of 2.44 x 10^6 and 2.41 x 10^6 cells/ml respectively. The TFC values obtained from the RF of Sheep 1 and 4 were over 2.5x more than those observed for Sheep 2 and 3.

Table 27 Main effect of different sheep rumen fluid on the total fungi cell count (cells / ml of RF)

Main effect of Sheep RF	**Total fungi count (TFC)**
1 (10^6 cells / ml)	7.69^a
2 (10^6 cells / ml)	2.44^c
3 (10^6 cells / ml)	2.41^c
4 (10^6 cells / ml)	6.53^b
SEM (10^6)	0.64

Means (n=3 and each n=1 was an average of 3 replicates) with different letters in the same column were significantly ($P < 0.05$) different; SEM, standard error of mean.

3.3.4 *Experiment 3: The effect of sheep RFs and forages on the TBC and TFC (cells / ml)*

The TBC and TFC (cells / ml) of both the buffered inoculum without forages and the buffered inoculum obtained after *in vitro* incubations with different forages for 48h are presented in Table 28 and 29 respectively. The microbial counts obtained from the buffered inoculum after incubations with different forages were higher than that recorded in the un-incubated buffered inoculum without forages. The forage types presented significant ($P < 0.0001$) effects on the bacterial and fungal counts. Among the grasses, legumes and forage controls, the highest TBC were recorded in *B. decumbens, L. Leucocephala* and *L. perenne* respectively. Among the grasses, legumes and forage controls, the highest TFC were recorded in *A. gayanus, L. Leucocephala* and *T. aestivum* respectively. The TFC produced by LL was approximately 6 times higher than the TFC value recorded in PP.

Table 28 Mean microbial counts of the un-incubated buffered inoculum before its use for the *in vitro* degradability after 48h of incubation

Direct count (cells/ ml)	Rumen fluid
Bacteria x 10^{10}	9.08
Fungi x 10^{5}	5.13

These values were used to correct the TBC and TFC obtained from the *in vitro* degraded forage samples

Table 29 Mean TBC and TFC in buffered inoculum after 48h of *in vitro* incubations with different forages

Microbial estimates	**Nigerian grasses**					**Legumes**			**Forage control**		
	PP	**PM**	**AG**	**BD**	**SEM**	**GS**	**LL**	**SEM**	**TA**	**LP**	**SEM**
Direct count (cells/ml)											
Bacteria x 10^{11}	1.22^{b}	1.54^{ab}	1.04^{b}	2.19^{a}	1.5 x 10^{10}	1.22^{a}	1.83^{a}	1.9 x 10^{10}	1.09^{a}	1.35^{a}	9.9 X 10^{10}
Fungi x 10^{5}	2.06^{b}	4.63^{a}	4.68^{a}	4.15^{a}	4.9 x 10^{4}	6.31^{b}	123.3^{a}	1.7 x 10^{5}	5.79^{bc}	2.81^{bc}	8.7 x 10^{4}

PP (*Pennisetum purpureum*), PM (*Panicum maximum*), AG (*Andropogon gayanus*), GS (*Gliricidia sepium*), LL (*Leucaena leucocephala*), TA (*Triticum aestivum*), LP (*Lolium perenne*). Means with different letters in the same row for each forage type are significantly ($P < 0.05$) different; SEM, standard error of mean.

3.4 Discussion

The main aim of this study extension was to determine the influence of each forage type (i.e. Nigerian grasses, legumes and forage controls) on the bacterial and fungal compositions of thawed RF (buffered inoculum) before and after their incubations for 48h as stated in chapter 2 and above. While the recording of protozoal counts would also have been helpful, it was not performed primarily due to the limited timeframe and resources that were allocated for this study. Indeed, any changes in bacterial and fungal counts were considered as potential indicators of changes in rumen microbial composition during *in vitro* incubations with selected forages for 48h in chapter 2. This microbial investigation was expected to complement the previously reported *in vitro* data in chapter 2, in order to select the two most suitable Nigerian grasses for their use, either alone or with a legume, to support better microbial growth. In order to achieve reliable microbial counts under a microscope, appropriate fixing and staining of microbes were needed. For this purpose, two concentrations of PFA (2% and 4%) and three staining agents (Acridine orange and SYBR gold for TBC, and lactophenol blue for TFC) were separately tested with the un-incubated thawed RF. This pre-investigation led to the choice of 4% PFA, and of SYBR gold and lactophenol as staining agents in the post-incubation RF for TBC and TFC respectively.

For logistical reasons, the rumen fluid samples used for the present experiments were frozen and thawed before being used for microbial investigation. However, there are reports in the literature that freezing and thawing affects the viability of bacteria and fungi, as freezing results in the solidification of cell membranes (Methé *et al.*, 2005) and formation of ice crystals that may damage the cells (Schimel and Clein, 1996; Brookman *et al.*, 2000; Schimel *et al.*, 2007; Jefferies *et al.*, 2010; Haei *et al.*, 2011). Also that different microbial groups (i.e. bacteria, fungi and protozoa) and species responds differently to freezing / stress due to the fact that they exhibits different growth pattern (Schimel *et al.*, 2007) and that some microbes do exhibit some physiological adaptation mechanisms (Ko *et al.*, 1994; Mindock *et al.*, 2001; Mihoub *et al.*, 2003; Bae *et al.*, 2004; Kandror *et al.*, 2004; Methé *et al.*, 2005; Walker *et al.*, 2006; Schimel *et al.*, 2007). In hindsight it would have been better not to freeze and thaw the rumen fluid samples. However, based on the short time of freezing which was 3 weeks (i.e. as shorter freezing time do affect the microbial physiology while longer freezing time affect the microbial community;Skogland *et al.* (1988)); the technique used for counting (i.e. the use of staining agents that is capable of counting both live and dead cells); not counting each group species (i.e. just having a total count of each group); and on the fact that cells dead through freezing do not necessarily lyse instantly (Skogland *et al.*, 1988). We assume that the total cell count for

each group can still be seen for counting and therefore posit that we can still compare the microbial results of the various grasses and legumes.

The slightly higher difference in TBC and TFC between the sheep RFs used for the incubation, and in any of the post incubation RFs that had the forages and its blank (un-inoculated RF), is a reflection that the sheep had been fed well before slaughter, and there is the possibility that the feed they were given was of better degradability. This supports the findings that nutrient availability in the rumen is dependent on the forage degradability, i.e. easily degraded feed in the rumen is capable of supplying the nutrients needed by rumen microbes for their growth and multiplication (Suharti *et al.*, 2011). However, the forage degradability is a reflection of the quality of the forage, i.e. good quality forage can be more quickly degraded in the rumen than the low-quality forage (Al-Soqeer, 2008; Njidda and Nasiru, 2010).

It was surprisingly to note differences in the TBC and TFC between different sheep, even though those sheep were raised at the same farm where they were reportedly offered the same diet. Nevertheless, these differences may be attributed to the pre-slaughter levels of feed intake (Belanche *et al.*, 2012), individual variations in microbial activity (Martínez *et al.*, 2010), host adaptation mechanisms (Henderson *et al.*, 2015), pre-fixation time (Srinivasan *et al.*, 2002), pre-slaughter handling and transport, as well as pre-slaughter dietary component selection (Jacobson *et al.*, 2002; Callaway *et al.*, 2003; Kannan *et al.*, 2014), and stage of feed degradability (Lee *et al.*, 2000b). Although the microbial composition seemed different, the sheep influence did not produce significant differences in the rumen fermentation metabolism (as shown in Chapter 2) during the *in vitro* fermentation of each forage type. This supports the statement of Warner (1962), that different animals receiving the same dietary regime and ration do show different rumen microbial populations but not the overall rumen fermentation metabolism.

The forage types (i.e. Nigerian grasses, legumes and forage controls) investigated influenced the rumen microbial (TBC and TFC) community composition differently and the influences were in line with the degradability studies (Chapter 2).The differences can be linked to the quality of the forages and their metabolites composition. Getachew *et al.* (1998); Ball *et al.* (2001); Dewhurst *et al.* (2001), and Belanche *et al.* (2012) identified that diet or feed (i.e. low-quality or high-quality feed) does alter the rumen microbial community composition differently. Low quality forages are not easily degraded (Wilson, 1994; Detmann *et al.*, 2009) and they support the growth of cellulolytic microbes, especially fungi (Bauchop, 1981; Ho and Abdullah, 1999; Krause *et al.*, 2003; Leis *et al.*, 2014). Good quality forages are easily degraded

(Cone and Becker, 2012; Cherdthong and Wanapat, 2013) and they tend to maintain a balance in the microbial community through an active synergistic interaction between microbes (Lee *et al.*, 2000b). Also, forages high in secondary metabolites are known to possess antimicrobial properties with much effect on protozoal and/or methanogens count (Tavendale *et al.*, 2005; Patra and Saxena, 2009; Tan *et al.*, 2011; Bodas *et al.*, 2012; Manasri *et al.*, 2012; Cieslak *et al.*, 2014; Piluzza *et al.*, 2014); the reduction in protozoal count is usually accompanied by an increase in other microbial (i.e. bacterial and fungal) counts (Getachew *et al.*, 1998; Ivan *et al.*, 2000; Suharti *et al.*, 2011; Cieslak *et al.*, 2016).

The Nigerian grasses, which are the main focus of this experiment, were of low quality and since they produced an appreciable degradability at 48h of incubation, it was justified to use the post-incubation RF at 48h for the microbial investigation. This is because degradability and digestibility are known to be stimulated by rumen microbes (Getachew *et al.*, 1998; Samanta *et al.*, 2001)}. Hence, it can be deduced that they were actively dividing and multiplying on the incubated forages at that time. Lengowski *et al.* (2016) found that the composition of the microbial community (i.e. bacteria and fungi) was more stable at 48h of incubation compared to earlier hours of incubation. Ho and Abdullah (1999) stated that low-quality forages were not readily degradable and they require longer retention times in the rumen for adequate microbial degradation to take place. Khan and Chaudhry (2011) also reported wide variations in chemical composition and *in vitro* degradability of low and high quality forages, suggesting possible links with variable rumen microbial activities.

The similar increased fungal count supported by three grasses (i.e. AG, BD, and PM) out of the four Nigerian grasses, indicates that these forages contained a more significant amount of structural carbohydrates, a substrate found in low quality forages that is needed for fungal colonization (Bauchop, 1979b; Bauchop, 1981; Edwards *et al.*, 2008). The expectation was that these forages would show higher fungal count, but PP was different. The decreased fungal count in PP can be attributed to lower fibre and lignin contents (Chapter 2), or it might be that it was supporting a synergistic relationship between microbes which led to its higher degradability (Lee *et al.*, 2000b) in the *in vitro* studies (Chapter 2).

Interestingly, the comparable increased TBC and TFC of BD in comparison with other grasses, despite its lowest CP content and fermentative characteristics (Chapter 2), indicates that forage plays a major role in altering the microbial community composition (Akin, 1989). There is also the possibility that the community of the increased TBC of the BD is composed of methanogenic bacteria, as it recorded higher methane content (Chapter 2), or that the fungal

metabolic activity led to the production of acetate (Bernalier *et al.*, 1991) that is required by methanogenic microbes for their growth and activity (Ho and Abdullah, 1999). The microbial composition of BD and PM were not in agreement with the report of Cheng *et al.* (1991), where higher bacterial count was complemented by an increase in various metabolic reactions which consequently supported better fibre degradability. This discrepancy supports the findings of Bernalier *et al.* (1992) and Bernalier *et al.* (1993) that anaerobic fungi do not degrade fibre efficiently when cellulolytic bacteria are present in abundance, due to the release of extracellular protein that inhibit fungal hydrolytic capability. The abundance of bacteria and fungi in rumen fluid with these 2 grasses might be because the protozoa, which were not counted in this study, were not adequately available. This is because protozoa are known to engulf and degrade other microbes, especially bacteria (Lengowski *et al.*, 2016), thus limiting the bacterial growth and activity that should have been involved in lignocellulose degradation (Gutierrez, 1958; Suharti *et al.*, 2011). However, their engulfing / degradation of bacteria is responsible for the increase in microbial protein turnover from the rumen to the abomasum (Wallace and McPherson, 1987; Fonty and Gouet, 1989).This suggests that protozoa were perhaps detrimental to the degradation of lignocellulose in the rumen. Lengowski *et al.* (2016) also reported a higher reduction in the protozoal population as early as 24h after initiation of incubation when the impact of several forages was investigated on the changes in the composition of the ruminal microbial community. The reduced TBC of AG, despite its high TFC and degradability (Chapter 2), supports the findings of Bernalier *et al.* (1993) that fungi degrade fibre efficiently when cellulolytic bacteria are not in abundance. The increased *in vitro* methane produced by AG among the grasses (Chapter 2), therefore aids in assuming that the available bacteria were not cellulolytic but methanogenic in function. The co-existence of the methanogenic bacteria and anaerobic fungi supported the fibre degradation (Marvin-Sikkema *et al.*, 1990; Bernalier *et al.*, 1992; Roger *et al.*, 1993). While it appeared from this study that these 3 forages can be used for further studies, it was decided to select the forages that supported the highest bacterial growth and high methane production (BD) and highest fungal growth and methane production (AG), despite variations in their *in vitro* degradability in chapter 2. These grasses were therefore used in further studies for their biological improvement in chapters 4-5.

Among the legumes, the highest TBC and TFC were found in *L. leucocephala,* even though it recorded the least fermentation characteristics but higher CP, TT and TP (Chapter 2). The higher bacterial and fungal counts may be attributed to the anti-methanogenic function of TT when present at a dose high enough to inhibit the growth of protozoa and associated methanogenic bacteria (Kreuzer *et al.*, 2009; Wang *et al.*, 2009; Tan *et al.*, 2011; Goel and

Makkar, 2012). The dose of plant metabolites in forages was discovered by Cieslak *et al.* (2016) as an essential factor altering the total bacterial count. An increase in bacterial count was discovered by Hsu *et al.* (1991); Ivan *et al.* (2000); Anantasook *et al.* (2013), as well as in fungal count (Romulo *et al.*, 1989; Ushida *et al.*, 1989), when protozoa were eliminated from the rumen. Protozoa are known to consume and digest bacteria (Wallace and McPherson, 1987; Lengowski *et al.*, 2016) and fungi (Ushida *et al.*, 1989). The obtained result is a reflection that the available bacteria might be cellulolytic bacteria, as lower ammonia and methane production and high pH (Chapter 2) were recorded with *L. leucocephala*. However, the co-existence of anaerobic fungi and cellulolytic bacteria has been found to restrict efficient cellulose or fibre degradation, which resulted in the lower fermentation characteristics of the forage (Bernalier *et al.*, 1992; Bernalier *et al.*, 1993). Therefore, LL was selected based on its ability to support microbial growth as a possible supplementary feed, with or without the selected grasses, in further studies.

Among the forage controls, TA supported higher fungal growth and lower bacterial growth while LP recorded lower fungal growth and higher bacterial growth that was comparable with TA. The differences can be attributed to the forage CP and structural components, which had a more significant influence on the nutrient degradability as shown in Chapter 2, the buffered inoculum used for the microbial investigation, and the forage class (i.e. C_3 and C_4) . Based on the microbial investigation, TA can be regarded as a low-quality forage because it recorded higher fungal count. Fungi are known to be an active coloniser of structural carbohydrates, i.e. fibre, a component found at higher levels in low-quality forages (Ho and Abdullah, 1999; Kumar *et al.*, 2014). Also, microbial growth and multiplication for possible degradation of low-quality forages are expected to be greater at longer incubation times (Cone *et al.*, 1997; Getachew *et al.*, 1998; Cone and Becker, 2012), which is evident in this study since the thawed RF used was from 48h of incubation. There was a clear indication that the mechanism by which microbes act or respond to C_4 (TA) and C_3 plant (LP) cell wall structures varies (Akin, 1989). The comparable TBC recorded could be that the C4 plant cell wall supports the growth of certain bacterial species to the detriment of other species (Lengowski *et al.*, 2016). The bacterial count was still lower than that for LP, despite recording higher fungal count. This supports the findings that efficient fibre degradability by fungi takes place when cellulolytic bacteria are not in abundance (Bernalier *et al.*, 1992; Bernalier *et al.*, 1993).

LP can be regarded as a good quality forage because it recorded a lower fungal count and higher bacterial count. The lower fungal count is an indication that the forage contained less structural carbohydrates that did not support fungal growth (Paul *et al.*, 2004; Kumar *et al.*, 2014). The

decreased TFC and TBC in the forage after 48h is an indication that the forages are no longer providing the microbes with energy, suggesting that the substrate has been exhausted well before 48h. This supports previous observations that good quality forages are easily degraded and exhausted after 10 to 20h of incubation (Cone and Becker, 2012; Cherdthong and Wanapat, 2013). Also, good quality forages are known to maintain a balance in the microbial community and an active synergistic interaction; this might have facilitated the higher degradation of some forages (chapter 2). Lee *et al.* (2000b) discovered better nutrient degradability when a synergistic relationship was found between fungi and bacteria. Therefore, based on the outcome of these results alongside the results of chapter 2, the forage controls can be used as positive and negative controls in further studies.

3.5 Conclusions

- It can be concluded that the use of SYBR gold with 4% PFA supported cell integrity which gave a better structural cell view for improved cell counting.
- Among the grasses, *A. gayanus* and *B. decumbens* showed that they were capable of supporting either highest bacterial or fungal counts, despite being low-quality forages as appeared in the *in vitro* studies (Chapter 2). This is an indication that these forages can be biologically improved, as they seemed to be supporting more microbial multiplication.
- *L. leucocephala* was shown to be capable of supporting microbial growth with the corresponding reduction in NH_3-N, methane and SCFA and nutrient degradability. This indicates that it can be used, along with other low-quality forages, to support the growth of microbes as well as to possibly reduce methanogens.
- *L. perenne* and *T. aestivum* showed that they could be used as positive and negative controls respectively. LP microbial composition was different from the Nigerian grasses, while that of TA corresponded well with those of the Nigerian grasses.
- Biological improvement of the selected forages, with or without *L. leucocephala* as a supplement, is needed for better ruminant growth and production.

Chapter 4

Investigation of Growing Conditions for *Pleurotus ostreatus* and *Ceriporiopsis rivulosus* before their Use to Upgrade Low-Quality Forages

4.1 Introduction

The tropical forages investigated in previous chapters have been found to be of low quality. This supports the existing literature that forages collected during the dry season in most tropical and sub-tropical countries are usually of low-quality, and they are not capable of meeting the nutrient requirements of the animals for performance and production (Odenyo *et al.*, 1997; Van Saun, 2006). Therefore, there is the need to upgrade these forages, with or without legume supplementation, by pre-treating them in order to degrade lignin that can limit the availability of carbohydrates (i.e. cellulose and hemicellulose) to ruminant animals. Lignin can be degraded by several pre-treatment methods, such as physical, chemical, physico-chemical and biological methods (Chaudhry, 1998; Alvira *et al.*, 2010; Chen *et al.*, 2010; Agbor *et al.*, 2011; Bajpai, 2016; Kumar and Sharma, 2017). However, the biological method has been the only method that adopts low-cost approaches that are not hazardous to the animals, people and the environment. The biological method involves the use of microbes, especially bacteria and fungi, in degrading lignin (Chaudhry *et al.*, 2001; Saritha and Arora, 2012; Sharma *et al.*, 2017) but fungi have been found to possess more powerful oxidative enzymatic function (Brown and Chang, 2014) and produce a more extensive lignin metabolism (Lotfi, 2014) than bacteria, thus making fungi more preferable in lignin degradation. Also, fungi attain higher lignocellulose degradation through mycelial penetration of plant tissues, and various extracellular enzymatic functions which penetrate complex plant structures and chemical bonds (Kirk and Cullen, 1998; Wong, 2009), especially under solid state fermentation (SSF) techniques (Acuña-Argüelles *et al.*, 1995; Pandey, 2003; Hölker *et al.*, 2004; van Kuijk *et al.*, 2016b).

However, different fungi vary in their enzymatic activity when presented with the same substrate for an upgrade. In this study, white rot fungi (WRF) were selected, as opposed to brown rot and soft rot fungi, due to their ability to degrade lignin but without affecting cellulose and hemicellulose contents (Zadražil and Brunnert, 1981; Blanchette, 1995; Keller *et al.*, 2003). The lignin degradation is expected to occur through the action of many ligninolytic enzymes, i.e. manganese peroxidase (MnP), lignin peroxidase, versatile peroxidase, aryl alcohol oxidase, glyoxal oxidase and laccase (Higuchi, 2004; Wong, 2009).

Various WRF use different ways to attack and decay lignocellulosic substrates; some do it non-selectively and some selectively. The selective WRF are capable of degrading lignocellulose with minimum organic matter loss, thus resulting in a higher availability of improved energy sources for animals (Blanchette, 1995). In this study, two selective WRF, a mushroom (i.e. *Pleurotus ostreatus*; PO) and a non-mushroom (i.e. *Ceriporiopsis rivulosus*; CR, also known as *Poria rivulosus* / *Obba rivulosus* / *Rigidoporus rivulosus* / *Physisporinus rivulosus*), were used as possible biological upgraders of the selected low quality Nigerian grasses (Chapter 3) without the legume. In addition, PO was selected because it has been extensively researched as a fungus used to upgrade crop residues and agro-industrial by-products (Hakala *et al.*, 2005; Assi and King, 2007; Fazaeli, 2007), and its mycelium has been found to be safe for ruminant feeding. On the other hand, CR was selected because it has not been extensively researched, and yet its use has been found to be harmless when consumed in a compound diet by animals over a short period of time (Okano *et al.*, 2009). However, there is the need to continually investigate fungal fermented substrates for mycotoxins / aflatoxin when new treatment is proposed as Sharma *et al.* (2012) was able to identify the presence of several mycotoxins in different fungal fermented wheat straw, although their presence was lower than 20ppb which is regarded as the permissible level in immature animals diet and poultry.

Fungal pretreatment begins with substrate inoculation with the use of an inoculum unit (IU). An inoculum unit can either be a spawn (i.e. mycelium coated grain) or a spore (i.e. agar plug). The spore is usually used on a small scale (Saxena *et al.*, 2001; van Kuijk *et al.*, 2015; van Kuijk *et al.*, 2016b), while the spawn is mostly used for large-scale upgrade, especially for mushroom production (Tripathi and Yadav, 1992; Sánchez, 2004). Since the spawn are usually larger than spores, they result in fewer inoculated sites when presented with the same quantity of inoculum as the spores over a particular period (van Kuijk *et al.*, 2015; van Kuijk *et al.*, 2016b). In this study, due to the small scale of the upgrade, the spores were intended to be used for the further upgrade. However, to obtain the spores that are capable of producing more fungal growth and sporulation as well as enzymatic function over an extended period of time, the optimal growth conditions that support this purpose need to be identified (Palmieri *et al.*, 2000; Krishna, 2005; Revankar and Lele, 2006; Patel *et al.*, 2009). Among these conditions, temperature and media have been found to affect the rate of fungal mycelial growth, sporulation and enzymatic function (Krishna, 2005; Magan, 2007; Hoa and Wang, 2015). Also, the incubation time has been identified to affect the rate and extent at which the fungi are capable of exhibiting their enzymatic functions, an essential or basic pre-requisite for fungal improvement of low quality feed (Arora and Sharma, 2009a). This current study therefore

investigated, without involving any cell wall components, different growth conditions (temperature, media, and incubation time) that supported extended fungal growth and laccase activity (Hattaka, 2001; Baldrian and Šnajdr, 2006) of *P. ostreatus* and *C. rivulos*us, before their use as inoculants for a subsequent fungal – substrate inoculation experiment in Chapter 5.

4.2 Materials and Methods

4.2.1 *Organisms and Culturing conditions*

Two aerobic fungal strains (*Ceriporiopsis rivulosus*, CR, and *Pleurotus ostreatus*, PO) were sourced from the Centre for Agriculture and Bioscience Interational (CABI), UK, as live cultures. The required culture media were purchased from Sigma-Aldrich. The fungal strains arrived in a slant tube (CR) and petri-dish (PO) respectively and, using a cork borer, mycelial discs (5mm mycelium diameter) of each fungus were taken and placed at the centre of the newly prepared plates of Potato dextrose agar (PDA) as described in section 4.2.2.1. The plates were kept in an incubator (MIR-554; 14020028, Panasonic cooled incubator) set at 25^0C for 7 days and then stored at 4^0C. Sub-culturing of fungi was done periodically for the maintenance of the fungal viability.

4.2.2 *Investigating conditions that facilitate optimum fungal growth and laccase production from the selected aerobic fungi*

The conditions that support the optimum growth and laccase production of PO and CR were investigated using three separate experimental designs, as described below. Experiment 1 involved a 2 (culture media) X 3 (temperature) X 9 (incubation time) factorial arrangement to examine the growth of CR on agar plates. Experiment 2 used a 2 (culture media) X 3 (temperature) X 16 (incubation time) factorial arrangement to investigate the growth of PO on agar plates. Experiment 3 followed a 2 (culture media) X 3 (temperature) X 3 (incubation time) factorial arrangement to study the laccase activity of each fungus in 2 different broths respectively. Further details of each of these three experiments are provided in the following sections.

4.2.2.1 *Effect of culture media, temperature and incubation time*

Two culture media (Potato dextrose and Malt extract) were used in both liquid (broth) and solid (agar) forms. Potato dextrose (4% w/v) and Malt extract (5% w/v) media were prepared in distilled water and then autoclaved at 121^0C for 15 minutes. The media, in the form of both agar and broth, were allowed to cool to about 45^0C before the relevant media were poured into either sterilised agar plates (~20ml/plate) or sterilised tubes (9ml/ tube). The agar plates were

then inoculated with 1 mycelial disc (5mm mycelium diameter) of each of the fungus types (CR and PO) respectively under aseptic conditions. The broth in tubes was inoculated under aseptic conditions with 2 mycelial discs (5mm mycelium diameter) of each fungus that were obtained from a 7 day old culture grown at 25^0C. The quadruplicated plates and tubes were then stored in an incubator at different temperatures (20^0C, 25^0C and 30^0C) over various incubation times. The selected incubation times for the fungi on agar plates were 1-9 days for CR and 1-16 days for PO, and in broth for both fungi were 7, 14, and 21 days. The fungal cultures were monitored daily on plates for mycelium diameter of CR until 9 days and PO until 16 days. The experiment ended when the plates were fully covered with each fungal mycelium. The experiment ended in the broth at each of the selected incubation times, when the contents of the tubes were filtered in order to obtain an enzyme extract, i.e. the filtrate from the inoculated and un-inoculated (control) tubes, for the determination of ligninolytic (i.e. laccase) enzymatic functions as described below.

4.2.3 *Measurements*

4.2.3.1 *Fungal mycelial diameter*

The mycelial diameter was measured in two directions at right angles to each other, from the first day of culture to 9 and 16 days when the plates were completely covered with CR and PO respectively. The mycelial diameter was measured for each culture medium, temperature and incubation time. The mycelial diameter was measured daily for its extension, using a transparent and sterilised ruler.

4.2.3.2 *Laccase assay*

The laccase activity was determined by monitoring the oxidation of the diammonium salt of ABTS (2, 2' – azino – bis (3 – ethylbenz – thiazoline – 6 – sulfonic acid), Sigma Aldrich Ltd) as a substrate (Geng *et al.*, 2004). In a reaction mixture (0.22ml) containing enzyme extract (0.02ml) and 5mM of ABTS dissolved in 10mM sodium acetate buffer (pH 5; 0.2ml), the absorbance was measured using a microplate reader (Spectra Max M3) set at 25^0C at 420 nm over a period of 10 mins. One unit of enzyme activity was defined as the amount of enzyme required for the oxidation of 1 µmol of ABTS per minute in the reaction mixture.

The laccase activity in U/ml was calculated using the extinction coefficient of ABTS (36000 M/cm) at a wavelength of 420 nm by the formula:

E.A. = (A*V)/ (t * e * v* l)

Where:

E.A. = enzyme activity

A = Absorbance at 420nm

V= Total volume of reaction mixture (ml)

t = Incubation time (min)

e = Extinction coefficient (M/cm)

v = Enzyme volume (ml)

l = path length

4.2.4 *Statistical analysis*

The data sets were statistically analysed by using the General Linear Model procedure in Minitab 16 software to determine the main and the interactive effects of culture medium, temperature and incubation time on the growth characteristics and laccase activity of the fungi. The effects were declared significant if $P < 0.05$.The means were separated for significance at $P<0.05$ by using Tukey's test. The data values were expressed as means ± SE (n = 4).

4.3 Results

4.3.1 *Interactive effects of culture media and temperature; culture media and incubation time; temperature and incubation time; and culture media, temperature and incubation time on C.rivulosus mycelium diameter growth.*

As presented in Tables 30 and 31, the interactive effects of culture media and temperature, culture media and incubation time;,temperature and incubation time, and media, temperature and incubation produced significant ($P < 0.05$) effects on CR mycelium diameter growth on plates. The media, i.e. (MEA and PDA), at each temperature presented an almost similar influence on the mycelium spread on the plates, with an increase with the increase in incubation time. However, MEA attained maximum mycelium spread on plates earlier than PDA.

The temperature of 30^0C led to the earliest attainment of maximum CR mycelium diameter (88mm) on plates at the 5^{th} day of inoculation, followed by 25^0C at the 7^{th} day, and lastly by 20^0C at the 9^{th} day. However, the highest temperature (30^0C) led to early drying up of the CR mycelium on plates, i.e. the higher the attainment of mycelium spread with the highest temperature, the faster the mycelium began to dry up on plates, therefore making 30^0C more favourable for shorter inoculation time and 20^0C for longer inoculation time. Also, at 20^0C the fungus was still seen to be growing, indicating that the growing phase did not reach a static phase.

Table 30 Interactive effects of Culture media (MEA and PDA) and Temperature (20^0C, 25^0C, and 30^0C); Culture media and Incubation time (0 – 9 days); and Temperature and Incubation time on the mycelium diameter growth of CR on plates.

	Culture media		
Temperature (^{0}C)	**MEA**	**PDA**	**SEM**
	Mycelium diameter (mm)		
20	43.35	41.15	3.67
25	55.90	56.50	3.54
30	63.83	65.28	3.28
	Culture media		
Incubation time (days)	**MEA**	**PDA**	**SEM**
	Mycelium diameter (mm)		
0	5.00	5.00	0.0
1	14.17^b	16.25^a	2.4
2	26.42^b	29.58^a	4.5
3	31.92^b	34.83^a	4.8
4	53.67	52.50	5.8
5	71.25^a	67.58^b	5.4
6	80.67^a	77.58^b	3.5
7	85.50	85.08	1.1
8	87.00^a	86.67^b	0.5
9	88.00	88.00	0.0

	Temperature (^{0}C)			
Incubation time (days)	**20**	**25**	**30**	**SEM**
	Mycelium diameter (mm)			
0	5.00	5.00	5.00	0.0
1	5.00^c	15.88^b	24.75^a	3.0
2	9.38^c	28.38^b	46.25^a	5.5
3	13.13^c	34.13^b	52.88^a	5.9
4	29.38^c	53.25^b	76.63^a	7.1
5	45.63^c	74.63^b	88.00^a	6.6
6	62.63^b	86.75^a	88.00^a	4.3
7	79.88^b	88.00^a	88.00^a	1.4
8	84.50^b	88.00^a	88.00^a	0.6
9	88.00	88.00	88.00	0.0

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM, standard error of mean. Media and temperature, SEM: 6.6; Media and incubation time, SEM: 3.6; and temperature and incubation time, SEM: 2.97.

Table 31 Interactive effects of Culture media (MEA and PDA), Temperature (20^0C, 25^0C, and 30^0C); and Incubation time (0 – 9 days) on the mycelium diameter growth of CR on plates.

Incubation time (days)	MEA Temperature (^{0}C)			PDA Temperature (^{0}C)			
	20	25	30	20	25	30	SEM
	Mycelium diameter (mm)						
0	5.00	5.00	5.00	5.00	5.00	5.00	0.0
1	5.00^{c}	14.25^{d}	23.25^{b}	5.00^{e}	17.50^{c}	26.25^{a}	4.2
2	9.75^{e}	26.50^{d}	43.00^{b}	9.00^{e}	30.25^{c}	49.50^{a}	7.8
3	13.50^{e}	31.50^{d}	50.75^{b}	12.75^{e}	36.75^{c}	55.00^{a}	8.4
4	32.75^{c}	52.00^{b}	76.25^{a}	26.00^{c}	54.50^{b}	77.00^{a}	10
5	48.00^{c}	77.75^{b}	88.00^{a}	43.25^{c}	71.50^{b}	88.00^{a}	9.3
6	66.00^{b}	88.00^{a}	88.00^{a}	59.25^{c}	85.50^{a}	88.00^{a}	6.1
7	80.50^{b}	88.00^{a}	88.00^{a}	79.25^{b}	88.00^{a}	88.00^{a}	2.0
8	85.00^{b}	88.00^{a}	88.00^{a}	84.00^{b}	88.00^{a}	88.00^{a}	0.9
9	88.00	88.00	88.00	88.00	88.00	88.00	0.0

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM, standard error of mean. Media, temperature and incubation time, SEM: 2.1.

4.3.2 *Interactive effects of culture media and temperature; culture media and incubation time; Temperature and incubation time; and media, temperature and incubation time on PO mycelium diameter growth on plates*

As shown in Tables 32 and 33, the interactive effects of culture media and incubation time, temperature and incubation time, culture media and temperature, and media, temperature and incubation produced significant ($P < 0.05$) effects on PO mycelium diameter growth on plates. The media, i.e. (MEA and PDA), presented a different influence on the mycelium spread on plates with an increase in incubation time; PDA led to the earlier attainment of maximum mycelium spread on plates than MEA.

The temperature of 25^0C led to the earliest attainment of maximum CR mycelium diameter (88mm) on plates at the 12^{th} day of inoculation, followed by 30^0C and 20^0C at the 16^{th} day respectively. This indicates that highest PO mycelium diameter growth on plates can be achieved at an earlier incubation time when grown at 25^0C, and at later incubation time when grown at 30^0C and 20^0C respectively.

The use of PDA at 25^0C, as well as MEA at 25^0C, led to the earliest attainment of maximum mycelium spread on plates at the 10^{th} and 12^{th} day respectively, followed by 30^0C at the 11^{th} day (PDA), then by 20^0C at the 15^{th} day (PDA), and lastly by 20^0C and 30^0C respectively at the 16^{th} day (MEA).

Table 32 Interactive effects of Culture media (MEA and PDA) and incubation time (0 - 16 days), and temperature (20^0C, 25^0C, and 30^0C) and incubation time, on the mycelium diameter growth of PO on plates.

	Media		
Incubation time (days)	**MEA**	**PDA**	**SEM**
	Mycelium diameter (mm)		
0	5.00	5.00	0.0
1	5.00	5.00	0.0
2	10.71[b]	14.08[a]	1.5
3	13.67[b]	16.79[a]	1.5
4	22.46[b]	25.83[a]	1.8
5	30.79[b]	35.58[a]	2.2
6	39.21[b]	46.63[a]	3.1
7	46.96[b]	58.38[a]	3.8
8	54.13[b]	67.63[a]	4.1
9	61.92[b]	74.79[a]	3.7
10	67.96[b]	80.50[a]	3.4
11	72.58[b]	82.58[a]	2.8
12	78.17[b]	84.08[a]	2.2
13	80.17[b]	85.00[a]	1.7
14	82.75[b]	87.25[a]	1.1
15	84.46[b]	88.00[a]	0.8
16	88.00	88.00	0.0

	Temperature (^{0}C)			
Incubation time (days)	**20**	**25**	**30**	**SEM**
	Mycelium diameter (mm)			
0	5.00	5.00	5.00	0.0
1	5.00	5.00	5.00	0.0
2	8.13[b]	17.94[a]	11.13[b]	1.8
3	10.75[c]	20.88[a]	14.06[b]	1.8
4	19.44[b]	31.50[a]	21.50[b]	2.2
5	28.50[b]	42.31[a]	28.75[b]	2.7
6	36.25[b]	55.56[a]	36.94[b]	3.8
7	44.38[b]	66.94[a]	46.69[b]	4.6
8	52.56[b]	75.88[a]	54.19[b]	5.0
9	62.19[b]	80.38[a]	62.50[b]	4.6
10	68.38[b]	83.63[a]	70.69[b]	4.2
11	72.63[c]	84.94[a]	75.19[b]	3.4
12	76.94[b]	88.00[a]	78.44[b]	2.7
13	78.56[c]	88.00[a]	81.19[b]	2.1
14	82.50[c]	88.00[a]	84.50[b]	1.3
15	85.44[b]	88.00[a]	85.25[b]	0.9
16	88.00	88.00	88.00	0.0

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM, standard error of mean. Media and incubation time, SEM: 2.7; and temperature and incubation time, SEM: 2.18.

Table 33 Interactive effects of Culture media and temperature, and culture media, temperature and Incubation time, on the mycelium diameter growth of PO on plates.

Temperature (^{0}C)	Media		
	MEA	PDA	SEM
	Mycelium diameter (mm)		
20	48.21	48.80	2.58
25	57.29	61.52	2.68
30	43.42^{b}	56.46^{a}	2.68

Incubation time (days)	MEA Temperature (^{0}C)			PDA Temperature (^{0}C)			SEM
	20	25	30	20	25	30	
	Mycelium diameter (mm)						
0	5.00	5.00	5.00	5.00	5.00	5.00	0.0
1	5.00	5.00	5.00	5.00	5.00	5.00	0.0
2	7.50^{c}	16.13^{a}	8.50^{bc}	8.75^{bc}	19.75^{a}	13.75^{ab}	2.6
3	10.75^{c}	18.75^{ab}	11.50^{c}	10.75^{c}	23.00^{a}	16.63^{b}	2.5
4	19.75^{c}	30.25^{ab}	17.38^{c}	19.13^{c}	32.75^{a}	25.63^{b}	3.2
5	27.88^{bc}	40.50^{a}	24.00^{c}	29.13^{bc}	44.13^{a}	33.50^{b}	3.9
6	36.13^{c}	52.13^{a}	29.38^{c}	36.38^{c}	59.00^{a}	44.50^{b}	5.4
7	44.38^{c}	60.50^{b}	36.00^{d}	44.38^{c}	73.38^{a}	57.38^{b}	6.5
8	52.38^{c}	68.63^{b}	41.38^{d}	52.75^{c}	83.13^{a}	67.00^{b}	7.1
9	62.13^{c}	76.00^{b}	47.63^{d}	62.25^{c}	84.75^{a}	77.38^{b}	6.5
10	69.38^{c}	79.25^{b}	55.25^{d}	67.38^{c}	88.00^{a}	86.13^{a}	5.9
11	73.50^{c}	81.88^{b}	62.38d	71.75^{c}	88.00^{a}	88.00^{a}	4.8
12	77.63^{b}	88.00^{a}	68.88^{c}	76.25^{b}	88.00^{a}	88.00^{a}	3.9
13	78.13^{b}	88.00^{a}	74.38^{c}	79.00^{b}	88.00^{a}	88.00^{a}	2.9
14	79.25^{c}	88.00^{a}	81.00^{c}	85.75^{b}	88.00^{a}	88.00^{a}	1.9
15	82.88^{b}	88.00^{a}	82.50^{b}	88.00^{a}	88.00^{a}	88.00^{a}	1.3
16	88.00	88.00	88.00	88.00	88.00	88.00	0.0

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM, standard error of mean. Media and temperature, SEM: 6.4; and media, temperature and incubation time, SEM: 1.5.

4.3.3 Effects of Culture media, Temperature and Incubation time on laccase activity (CU/ml) of CR using a liquid medium

As presented in Table 34, the culture media, temperature and incubation time recorded significant ($P < 0.05$) effects on the laccase activity of CR, with PDB, 20^0C and 21st day recording the highest laccase activity values (5.47, 6.66 and 5.98 CU/ml) respectively. However, the laccase activity produced at the 21st DOI was not significantly ($P < 0.05$) different from that of the 14th DOI. The results showed that as the temperature increased, the laccase activity of CR reduced, while as the incubation time increased, the laccase activity increased irrespective of the culture media.

As presented in Figure 4, the interactive effects of culture media, temperature and incubation time recorded significant ($P < 0.05$) influences on laccase activity of CR in broth. CR cultured in PDB at 20^0C for 21 days recorded the highest activity. This indicates that the use of PDB at a lower temperature was capable of increasing the laccase activity of CR for a longer inoculation time. In most cases, the laccase activity at the various temperatures increased with an increase in the incubation time. However, a reduction in laccase activity was obtained after 14th DOI when CR was grown using both media at 30^0C. This indicates that higher temperature was detrimental to CR exhibiting its laccase activity.

Table 34 Main effects of Culture media (MEA and PDA), Temperature (20^0C, 25^0C, and 30^0C) and Incubation time (0 – 9 days) on laccase activity (CU/ml) of CR using liquid medium

Culture media	**Laccase (CU/ml)**
MEB	3.32^b
PDB	5.47^a
SEM	0.8
TEMPT (^{0}C)	**Laccase (CU/ml)**
20	6.66^a
25	4.25^b
30	2.29^c
SEM	1.0
Incubation time (days)	**Laccase (CU/ml)**
7	1.85^b
14	5.37^a
21	5.98^a
SEM	1.0

Means with different letters in the same column for each factor effect were significantly ($P < 0.05$) different; SEM, standard error of mean.

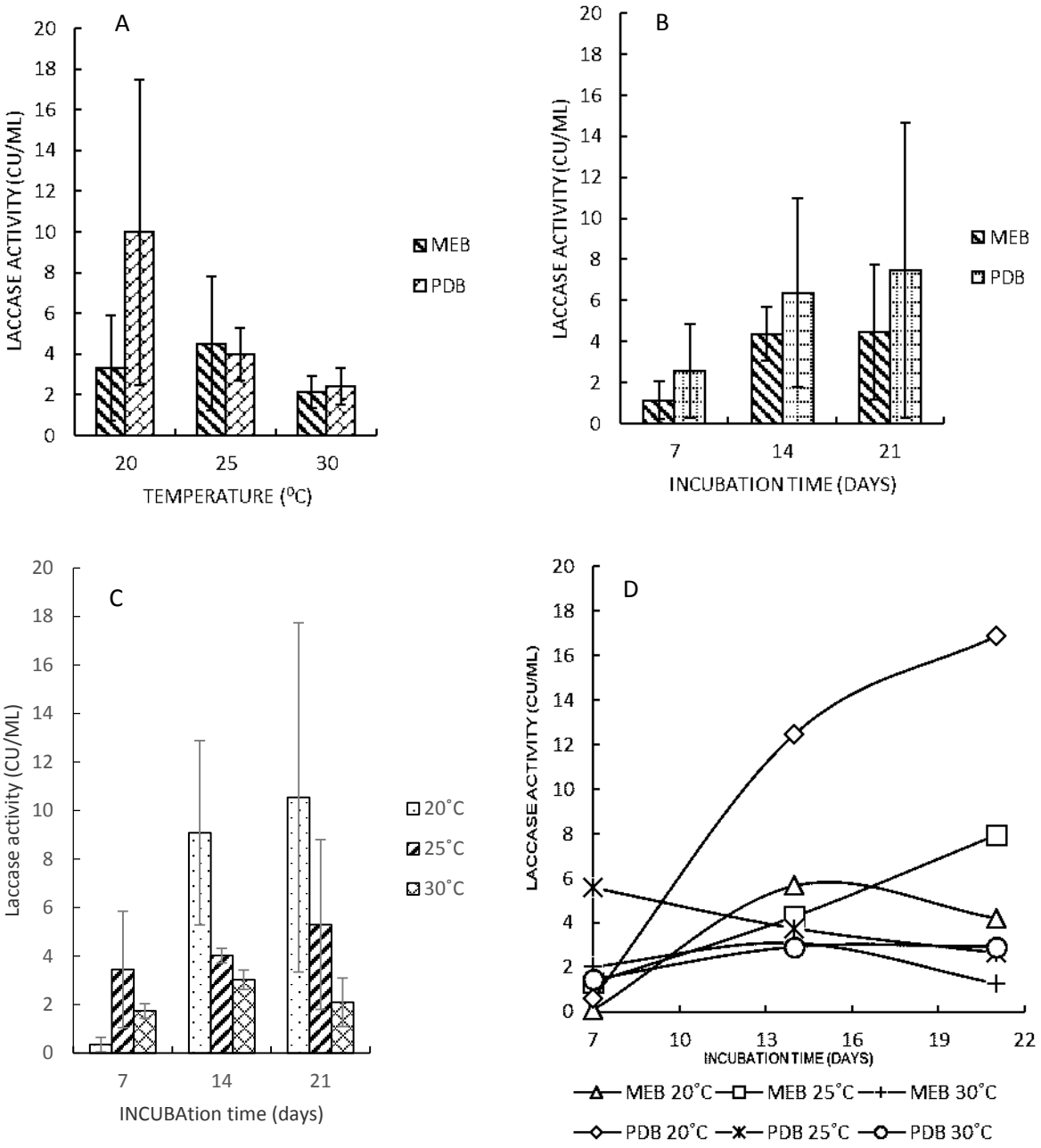


Figure 4 Interactive effects of A) Temperature and Culture media, B) Incubation time and culture media, C) Incubation time and Temperature D) Media, temperature and incubation time on intracellular laccase activity (CU/ml) of CR using liquid culture media (Malt extract (MEB) and Potato dextrose (PDB) broths. (Error bars represent ± standard deviation from appropriate means).

4.3.4 *Effects of Fungi, Culture media, Temperature and Incubation time on laccase activity (CU/ml) of PO using a liquid medium*

As shown in Table 35, the culture media, temperature and incubation time recorded significant ($P < 0.05$) effects on the laccase activity with MEB, 30^0C and 14th day recording the highest values (22.46, 20.06 and 17.83 CU/ml) respectively. The results showed that as the temperature increased, the laccase activity increased, while as the incubation time increased, the laccase activity increased until the 14th day, with a subsequent slight reduction in activity at the 21st DOI.

As presented in Figure 5, the interactive effects of culture media, temperature and incubation time recorded significant ($P < 0.05$) influences on laccase activity of PO in broth. The PO cultured in MEB at 30^0C for 21 DOI recorded the highest activity. This indicates that the use of MEB at a higher temperature was capable of increasing the laccase activity of PO for a longer inoculation time. The laccase activity at the various temperatures increased with increase in the incubation time up to the 14th DOI, after which the laccase activity reduced, except for PO grown on MEB at 30^0C. This indicates that a higher temperature was not detrimental to PO exhibiting its laccase activity when grown on MEB for a longer inoculation time.

Table 35 Main effects of Culture media (MEB and PDB), Temperature (20^0C, 25^0C, and 30^0C) and Incubation time (0 – 16 days) on laccase activity (CU/ml) of PO using liquid culture media

Culture media	**Laccase (CU/ml)**
MEB	22.46[a]
PDB	17.87[b]
SEM	2.6
Temperature (^{0}C)	**Laccase (CU/ml)**
20	20.06[b]
25	13.32[c]
30	27.10[a]
SEM	3.2
Incubation time (days)	**Laccase (CU/ml)**
7	16.84[b]
14	25.82[a]
21	17.83[b]
SEM	3.2

Means with different letters in the same column for each factor effect are significantly ($P < 0.05$) different; SEM, standard error of means.

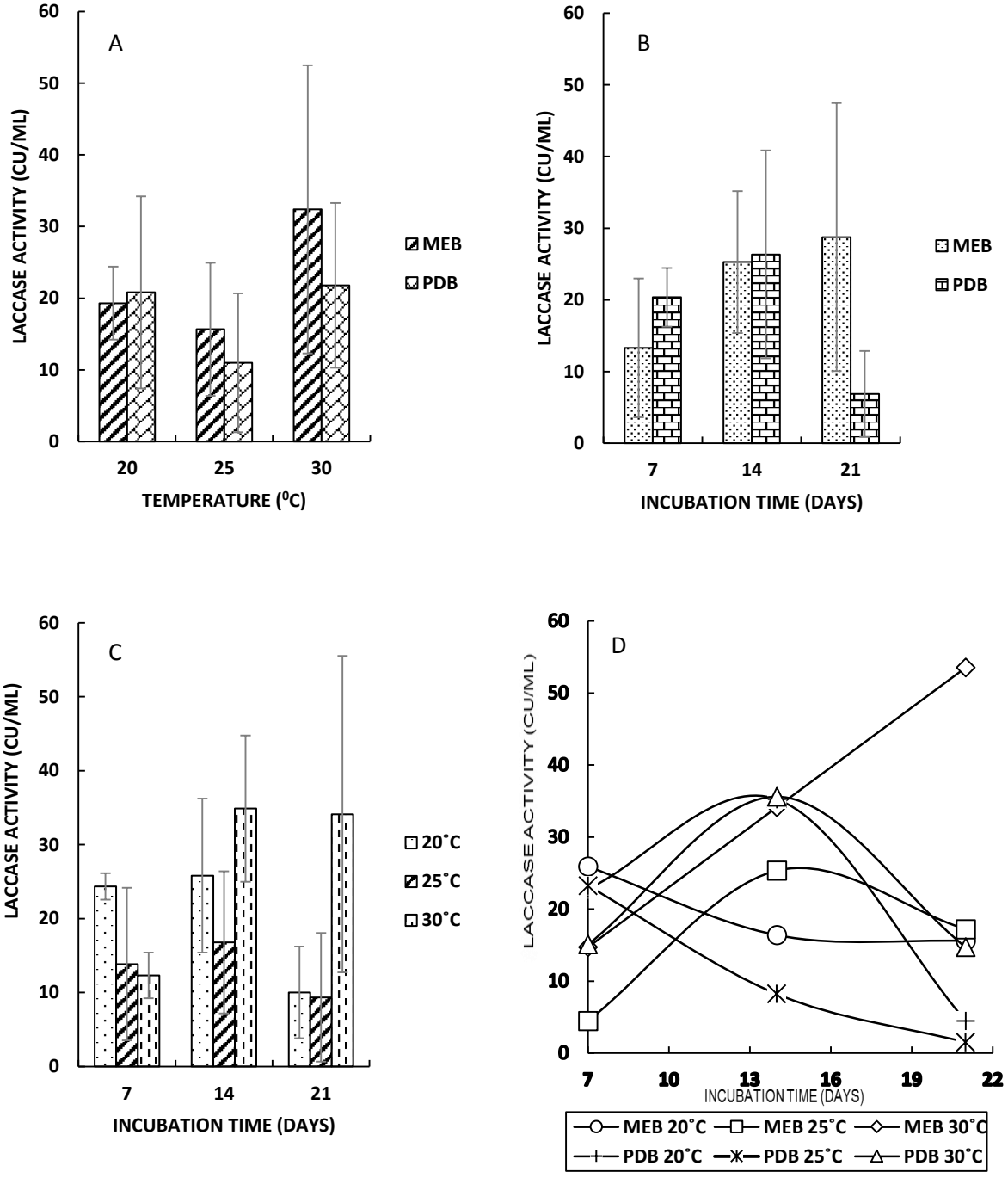


Figure 5 Interactive effects of A) Temperature and Culture media, B) Incubation time and culture media, C) Incubation time and Temperature D) Media, temperature and incubation time on intracellular laccase activity (CU/ml) of PO using liquid culture media (Malt extract (MEB) and Potato dextrose (PDB) broths. (Error bars represent ± standard deviation from appropriate means)

4.4 Discussion

This study was carried out to investigate some growth conditions (i.e. media, temperature and incubation time) that support the growth and sporulation, and laccase enzymatic function of PO and CR over extended periods of time. This pre-requisite information was needed to identify the best plates from which to obtain actively growing agar plugs for use for the forage substrate inoculation in a subsequent study. The growth conditions to be identified must be capable of supporting the growth and enzymatic function of the fungi over a longer period, because the fungal upgrade of lignocellulosic substrates is found to occur at longer fermentation periods (Zhang *et al.*, 2015).

In this study, the use of a lower temperature (20^0C) with potato dextrose agar supported the mycelial growth and sporulation, as well as laccase activity, of CR over a longer period. Although a higher temperature, such as 28^0C, tended to support early and faster growth of CR, as recorded by Hakala *et al.* (2004), Hatakka *et al.* (2003) found 25-30^0C as the most preferred temperature range required for CR and some other fungi when grown on ME agar. However, the higher temperature was detrimental to the fungus exhibiting its laccase activity (Patrick *et al.*, 2011), an activity that was required to be maximized for lignin to be properly degraded. This indicates that the temperature was high enough to dry up the fungus and also might have disrupted the fungal metabolic process by denaturing essential enzymes (Jonathan and Fasidi, 2003), which might give an insignificant effect on lignin or fibre degradation if used for the fungal – substrate inoculation in later studies. Also, the high temperature might be affecting fungal sporulation, since laccase has been found to be involved in fungal sporulation (Thurston, 1994). This supports the contention that obtaining the optimal temperature was very important, due to its impact on growth and sporulation and exhibition of enzymatic activity (Patel *et al.*, 2009). Therefore, the use of 30^0C was only favourable for attaining higher mycelial diameter over a shorter period. This indicates that, at this temperature, if the fungi are required for biodegradation of lignin, the fungal growth might get into a declining phase earlier. However, for fungi to be used in obtaining possible lignin biodegradation, a longer fermentation cycle is required, which is one of the drawbacks of using fungi for forage upgradation (Zhang *et al.*, 2015). Therefore, a temperature that supported an increase in CR mycelial diameter spread, as well as laccase activity over an extended period, was preferred for possible lignin biodegradation in the selected forages. In such a case, 20^0C will be the most preferred temperature, followed by 25^0C and lastly 30^0C.

The use of PDB led to an increase in CR laccase activity; this showed that the media provided the required nutrients needed by the fungi to perform the necessary enzymatic function. It has

been reported that laccase production by CR required nutrient-rich medium or rich culture conditions for its optimum activity (Galhaup *et al.*, 2002; Hakala *et al.*, 2006). In addition, PDB, being very rich in nutrients, was able to support higher laccase activity and was considered to be most preferable (Chang, 1999). Therefore, agar plugs can be obtained from plates grown at 20^0C on PDA as inoculum for the low-quality forages, and the same growth conditions were planned to be used for the fungal-substrate inoculation in the subsequent chapter.

In this study, the use of a higher temperature (30^0C) with malt extract agar (MEA) supported the mycelial growth and sporulation, as well as laccase activity, of PO over a longer period of time. Although 25^0C tended to support early and faster mycelial extension of PO in PDA medium, which was in agreement with the reports of Zadrazil (1976), Azmi and Seppelt (1997), Zharare *et al.* (2010) and Sardar *et al.* (2016), it did not agree with the findings of Kashangura (2008) and Hoa and Wang (2015), who recorded 28^0C as the preferred temperature to grow *Pleurotus* spp on agar plates. The differences may be attributed to the type of *Pleurotus* spp, isolate type and or strain, which respond differently even when they are grown under similar or different conditions (Kurtzman and Zadrazil, 1982; Jalc *et al.*, 1997; Mata and Savoie, 1998; Membrillo *et al.*, 2008; Zharare *et al.*, 2010; Sardar *et al.*, 2016; Nayan *et al.*, 2017).

However, 25^0C did not support as high laccase activity as was recorded with the use of 30^0C and 20^0C. This might be because 25^0C was only supporting the mycelial elongation at the expense of sporulation, as was experienced when grown at 30^0C and 20^0C. The obtained higher laccase activity at 30^0C was either in agreement, or close to, the findings of Patel *et al.* (2009); Patrick *et al.* (2011), Novotný *et al.* (2001), Palmieri *et al.* (2005), and Zadrazil *et al.* (1999a), where 30^0C, 28^0C, 28^0C, 28^0C, and 30^0C were reported as the optimum temperatures for *Pleurotus* spp laccase activities, respectively, when these temperatures were examined under solid state and submerged fermentation.

Therefore, the use of 25^0C was only favourable for attaining higher mycelial diameter for a shorter period. This indicates that, at this temperature, if the fungi are required for biodegradation of lignin, the fungal exhibition of laccase activity might be slowed down. However, for fungi to be used in obtaining possible lignin biodegradation, a longer fermentation cycle is required, which is one of the drawbacks of using fungi (Zhang *et al.*, 2015). Therefore, a temperature that supports an increase in PO mycelial diameter spread, as well as laccase activity over an extended period, will be preferred for possible lignin biodegradation in the selected forages. In such cases, 30^0C will be the most preferred option which produced a comparable response regarding mycelial growth at 20^0C, and lastly by 25^0C.

The use of MEA produced increased mycelial growth and laccase activity over an extended period. This supports the fact that the higher nutrient content of PDA in some cases produced higher mycelial growth at the detriment of sporulation, while in some cases a less rich medium has been found to support enzymatic functions (Stamets and Chilton, 1983). In addition to this, laccase has been found to be involved in sporulation by fungi (Thurston, 1994). Malt extract is made up of tyrosine and tryptophan (aromatic amino acids), which produce metabolites which act as oxidative mediators. The mediator causes a reaction which catalyses improved laccase activity (Cai *et al.*, 1993; Eggert *et al.*, 1996; Zhao and Kwan, 1999; Arora and Gill, 2001; Hatakka and Hammel, 2011). Therefore, agar plugs can be obtained from plates grown at 30^0C and/or 20^0C on MEA as inoculum for the low-quality forages, and the same growth conditions were used for the fungal-substrate inoculation in the subsequent chapter.

4.5 Conclusions

The major conclusions drawn from this study are listed below:

- The CR agar plugs can be obtained from the fungus grown on PDA at 20^0C, as these growth conditions supported the growth and laccase activity of the fungus over an extended inoculation time. The same conditions can subsequently be used for the CR inoculation of the selected low-quality forages as a possible biological upgrader in the next chapter (Chapter 5).

- The PO agar plugs can be obtained from the fungus grown on MEA at 30^0C and/or 20^0C, as these growth conditions supported the growth and laccase activity of the fungus over an extended inoculation time. The same conditions can subsequently be used for the PO inoculation of the selected low-quality forages as a possible biological upgrader in the next chapter (Chapter 5).

Chapter 5

Influence of loss of solubles on the ability of Aerobic Fungi to improve the Nutritive Quality of Pre-treated Forages

5.1 Introduction

The pre-treatment of low quality forages with aerobic fungi (i.e. selective white rot fungi) as a means of improving their nutritive value (i.e. chemical composition and digestibility) for ruminant feeding has received more attention over the years (Yu *et al.*, 2009a; Arora and Sharma, 2011; Abdel-Hamid *et al.*, 2013; Mahesh and Mohini, 2013; van Kuijk *et al.*, 2015; Nayan *et al.*, 2018; Niu *et al.*, 2018). Among these white rot fungi, *P. ostreatus* and *C. rivulosus* were selected for this study (as discussed in Chapter 4) based on their use and perceived ligninolytic ability to upgrade agro-industrial by-products and crop residues as reported by several researchers (Tuyen *et al.*, 2013; Nayan *et al.*, 2017; Nayan *et al.*, 2018; Niu *et al.*, 2018), although these fungi have not been tested to upgrade mature grasses as ruminant feeds.

In improving the forages by the fungi, the fungi requires conditions that support their growth and activity, some of which were identified in previous chapter (Chapter 4). However, in some cases, no improvement was achieved despite using conditions that support the fungal growth and activity. This leads some researchers to question the ability of these fungi in improving the pre- treated substrates. This query was based on several factors such as substrate type (Tuyen *et al.*, 2013; van Kuijk *et al.*, 2015), fungal strain and species (Jalč *et al.*, 1994; Akinfemi, 2010b; Cragg *et al.*, 2015; van Kuijk *et al.*, 2015; Nayan *et al.*, 2017), extent of degradation (Capelari and Zadrazil, 1997; Akinfemi, 2010b) and release of anti-nutrients as toxins or metabolites by these fungi (Reid, 1989; Capelari and Zadrazil, 1997; Ramirez-Bribiesca *et al.*, 2011; Sharma and Arora, 2015) which affect palatability (Mahesh and Mohini, 2013), chitin content (Arora and Sharma, 2009a) and extensive fermentation of structural carbohydrates with increased ash content (Jung *et al.*, 1992; Karunanandaa *et al.*, 1995; Sharma and Arora, 2010a; Mahesh and Mohini, 2013; Sharma and Arora, 2015). Most of these factors do have a major influence on the intake, digestibility and/or degradability of the pre-treated substrates (Mahesh and Mohini, 2013). However, no information is available on the impact that loss of water soluble carbohydrates (WSC) and neutral detergent solubles (NDS) may have on the degradability/ digestibility of fungal pre-treated substrates.

The WSC and NDS usually accompany lignocellulose degradation, as they are by-products of the degradation process and they are released when the growth conditions are optimal for fungal growth and enzymatic function. To enable the liberation of solubles, further different growth conditions (i.e. substrate-liquid ratios and extended inoculation time) were combined with the ones identified in Chapter 4. The combined use of the growth conditions was based on the fact that the optimum incubation length may be different for different substrate-liquid ratios. The selected growth conditions were used as they play a major role in fungal growth and activity. Some researchers have questioned the involvement of these products in *in vitro* digestibility, but they are of the opinion that they are involved in increasing the nutritive value and initial fungal mycelial growth, but with no involvement in digestibility (Arora and Sharma, 2009a). Some researchers have failed to establish a relationship between fibre degradation, straw digestibility and water-soluble contents (Rolz *et al.*, 1986), while others suggest that they contribute to an increased digestibility (Sharma and Arora, 2015; Zuo *et al.*, 2018). However, their influence on degradability/ digestibility has not been ascertained or investigated.

In order to investigate the influence that the loss of these soluble compounds will have on degradability, this experiment employed two filtration methods: the pump filtering method (PFF, which represents the situation when the liquids are pressed out of the fungal treated forages by heavy stones that the farmers use to cover the fungal pre-treated forage sacks on the farms) and the free flow filtering method (FFF, which represents cases when the liquid freely drains out of fungal treated substrates in sacks on farm). This study, therefore, evaluated the influence that loss of solubles has on the ability of the fungi to improve the nutritive quality of selected forages.

5.1.1 *Objectives*

To investigate the fungal growth and laccase activity, as well as the influence of soluble losses (using PFF and FFF methods), on the ability of two fungi *(C. rivulosus;* 20^0C and *P. ostreatus;* 30^0C) to improve the nutritive quality of pre-treated forages (*A. gayanus*, *B. decumbens*, *L. perenne* and *T. aestivum* straw) using two different substrate: liquid ratios (1:3 and 1:5) over two different inoculation times (14 and 28 days).

5.1.2 *Specific objectives*

To determine the culture conditions (i.e. substrate-liquid ratio and inoculation time) that support increased fungal activity by determining the laccase enzymatic activity. Also, to

investigate the influence of loss of solubles due to two filtration methods on the ability of the fungi to improve the following:

- Proximate (dry matter, organic matter, crude protein, ash, carbon) and fibre composition involving neutral detergent fibre (NDF), acid detergent fibre (ADF) and ADL as determined by using regression equations derived by using the detergent fibre composition data of the pre-treated forages in Chapter 2.
- The secondary metabolites (total phenols, total tannin) and total antioxidant capacity of the pre-treated forages using chemical analyses.
- *In vitro* dry matter (IVDMD) and organic matter (IVOMD) degradability, NH3-N, CH4, volatile fatty acids (VFA), pH, gas production of the pre-treated forages after filtration with only the PFF method. This method was more preferred as it produced more uniform and consistent soluble loss in the pre-treated forages which serve as a source of more insoluble substrate from which the pronounced effect of soluble loss can be examined in the *in vitro* studies.

5.2 Materials and Methods

5.2.1 *Aerobic fungus source for substrate inoculation*

Agar plugs of the aerobic fungi (*Ceriporiopsis rivulosus* and *Pleurotus ostreatus*) were obtained from plates cultured at the growth conditions ascertained in Chapter 4, i.e. Potato dextrose agar at 20^0C (CR) and Malt extract agar at 30^0C (PO) for 10 days respectively. The length of incubation used in Chapter 4 was for a total period of 21 days, but the choice of using a slightly longer incubation time (i.e. 28 days) in this study was based on the fact that it involved the use of structural substrates (i.e. forages) that require more time for fungi to degrade, and also based on the time frame used in published papers when samples with smaller particle sizes were used. For the inoculation of the forages, 3 pieces of 5mm mycelial discs /agar plugs were used in these studies.

5.2.2 *Preparation of forage substrates and inoculation with aerobic fungi*

A 4 (forages) x 2 (substrate: liquid ratio) x 3 (fungal treatment) factorial arrangement at each inoculation time (14 and 28 days) for each filtration method (i.e. PFF and FFF) was used in this study. The samples consisted of the two Nigerian low-quality forages (*B. decumbens* and *A. gayanus*) and two forage controls representing either very low quality straw (*T. aestivum* straw) or moderate quality temperate grass (*L. perenne*). On this basis, samples of 5g per substrate (ground through 2-mm screen) were accurately weighed into 250 ml Erlenmeyer flasks that were thoroughly washed and oven dried for 10 minutes at 100^0C. The weighed samples in various flasks were rehydrated with two different amounts (15ml and 25ml) of 1% Potato dextrose broth (CR) and 1% malt extract broth (PO) respectively to set the solid/liquid ratio. The flasks were immediately covered with wool, wrapped with foil paper and autoclaved (Ensign Autoclave; Rodwell Scientific Instrument; EN 837-1) at 121^0C for 15mins. Each treatment was replicated thrice. Each of the Erlenmeyer flasks was then inoculated with three 5mm mycelial discs exactly at the centre of the substrate to prevent it from touching the sides of the bottle and was immediately covered with wool & foil. The inoculation of the forages was carried out in a Class II microbiological safety cabinet (Envair; BS 5726 1992; din 12950 1990; nfx 44 1984), and the flasks were thereafter incubated (Panasonic; MIR-554-PE; cooled incubator) at either 20^0C (CR) or 30^0C (PO) in a dark environment at 95% humidity. Replicated control flasks containing the substrates and the media but no fungal mycelium were also autoclaved. Each filtration method had seventy-two flasks made from the combinations of forage, substrate to liquid ratio and fungal treatment in which thirty-six flasks were processed after 14 and 28 days of incubation along with control flasks.

5.2.3 *Processing of inoculated substrates for laccase enzymatic activity*

A 4 (forages) x 2 (substrate: liquid ratio) x 3 (fungal treatment) factorial arrangement at each inoculation time (14 and 28 days) was used for the laccase study and each treatment was replicated thrice. The supernatant obtained from samples assigned for each filtering method were pooled together for determination of laccase activity as they both undergo the same process and timing. After each inoculation time, 25ml of 10mM sodium acetic buffer solution (pH 5.0) was added to each selected flask for extraction of enzymes. The flasks were then kept in a shaking incubator (200rpm) for 20mins at 20^0C. The contents of each flask were filtered through a dried what-man filter paper no. 1 (150 mm diameter) and the filtrate was centrifuged at 10,000 rpm at 4^0C for 20 min. The supernatant was collected for its use for enzyme determination (Sharma, 2011; Arora et al., 2002).

5.2.4 *Processing of the substrates for the evaluation of loss of neural detergent solutes and water-soluble contents alongside nutritive contents and in vitro digestibility*

After the collection of the filtrate, the flasks from which the solids on the filter paper were obtained were then split equally to two different parts. Half of the flasks with their solids on the filter paper were used as samples obtained from free-flow filtering under gravity (i.e. FFF) without any pressure, while the other half of the flasks with their solids on the filter paper were used as samples obtained from pump filtering with a vacuum pump (i.e. PFF). The solid contents for the PFF method were washed with 30ml of sterile boiling water to remove the mycelia as well as the water-soluble contents; afterwards the added water was removed from the washed solids by light suctioning using a pump. The solid contents for the FFF method were allowed to drain their liquids without adding any water as described in **5.2.3**. The contents of each PFF or FFF method, alongside the filter papers, were then dried at 60^0C and subsequently used for various analyses, i.e. chemical analysis, antioxidant analysis and *in vitro* analysis (as described below). Loss of dry matter and other nutrients as influenced by fungal inoculation was calculated as the differences in absolute weight between the control (un-inoculated substrate) and the inoculated substrates and the results were presented as percentages of the relevant nutrients of the untreated controls.

5.2.5 *Measurements*

5.2.5.1 *Determination of laccase activity (enzymatic activity)*

Laccase activity was determined by monitoring the oxidation of the diammonium salt of ABTS (2, 2' – azino – bis (3 – ethylbenz – thiazoline – 6 – sulfonic acid, Sigma Aldrich Ltd) as a substrate (Geng *et al.*, 2004, see Chapter 4 for details).

5.2.5.2 *Determination of the Chemical composition of the dried fungal degraded samples obtained from pump assisted or free flow filtration of forages*

The chemical components (DM, OM, Ash, CP and carbon); fibre components (NDF, ADF); and secondary metabolite contents (TP, TT and TA) were determined as described in Chapter 2. Carbon values were obtained along with the Nitrogen values using the Elementar vario macro cube analyser, as previously described. The forages were not chemically analysed for ADL content but these were calculated from the regression equations obtained for ADL vs ADF data sets already presented in Chapter 2 (see regression equation below). The analytical method for lignin analysis was not used as this is time consuming, laborious, expensive, and produces more acidic waste (Van Soest, 1964; Gomes *et al.*, 2011). In addition, none of the available analytical methods for lignin estimation have been standardized or have produced the most accurate estimate for lignin, as they tend to give different results (Hatfield and Fukushima, 2005; Gomes *et al.*, 2011). Based on these reasons, ADL values were predicted from fibre content, i.e. acid detergent fibre (see Figure 6), as predicted values using empirical models and equations have previously been used in literature to derive energy contents, fibre contents, intake and digestibility parameters (Traxler *et al.*, 1998). Although, these are not very precise, they provide an indication of what might be expected and the obtained ADL values (especially with the FFF method) were closer to that obtained in Chapter 2 and can therefore be used to justify the main goal. However, analytical estimation of ADL may be more helpful and crucial for lignin determination in future studies of this kind.

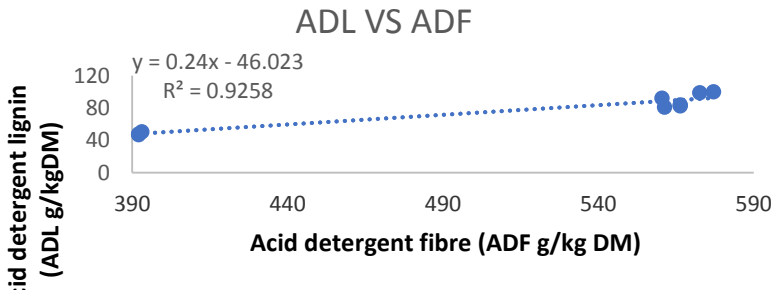


Figure 6 :A graph reflecting the regression equation generated from ADL/ADF values from Chapter 2 needed for generating ADL in Chapter 5

5.2.5.3 *Determination of the in vitro degradability of the dried fungal degraded samples obtained from pump or free flow filtered forages*

After the determination of the chemical composition, the best substrate-liquid ratio in each processing method (i.e. PFF and FFF) was identified as 1:5 based on its ability to influence more nutrient loss in the treated forages. Samples from this ratio were used for the IVD of the degraded dried residues using Menke and Steingass (1988) procedures (as described in Chapter 2) but only that of the PFF was determined. This is because this method recorded greater soluble losses and appeared to show more consistency in the amount of liquid removed due to the pump assisted filtration. Therefore these insoluble filtered residues seemed more preferable for testing the effect of soluble loss on the *in vitro* degradability. The buffered inoculum used for the in vitro studies was analysed for it fermentation parameters and was composed of 24.98 mg/L ammonia, 53.36 mmol/l (tVFA), 33.12 mmol/l (acetate), 12.54 mmol/l (propionate), 6.70 mmol/l (butyrate), 0.57 mmol/l (isovalerate), 0.44 mmol/l (valerate) contents and pH (6.99). The percentage of CH4 /TGP was calculated.

5.2.6 *Statistical analysis*

The data for laccase activity were statistically analysed by following the factorial design according to the Generalized Linear Model procedure in Minitab 16 software to determine both the main and interactive effects of each fungal treatment, forage and substrate: liquid ratio on the laccase activity at each of the inoculation times (14 and 28 days). The data for chemical composition were statistically analysed by following the factorial design according to the Generalized Linear Model procedure in Minitab 16 software to determine the influence of filtration method (i.e. PFF and FFF) alongside the main and interactive effects of fungal strain, substrate, and substrate: liquid ratio on the chemical composition and nutrient losses at each of the inoculation times (14 and 28 days). $P < 0.05$ was considered to indicate a statistically significant effect on each of the tested parameters and different means were separated using Tukey's test. Each value is expressed as mean ± SE (n = 3).

In terms of the *in vitro* studies, a factorial design using the Generalized Linear Model procedure on Minitab 16 software was used, for samples from the PFF method, to determine the main and interactive effects of fungal strain, forages, and time of inoculation on IVDMD, IVOMD and fermentation parameters (VFAs, CH4, NH3-N, pH, and total gas production). A $P < 0.05$ was considered to indicate statistical significance and means were separated using Tukey's test. Each value is expressed as mean ± SE (n = 3).

5.3 Results

5.3.1 *Laccase activity of the fungi*

The main effects of fungus (*C. rivulosus* and *P. ostreatus*), forage (*A. gayanus*, *B. decumbens*, *L. perenne* and *T. aestivum*) and substrate: liquid ratio (1:3 and 1:5) on the laccase activity after fourteen days of inoculation are presented in Table 36. The main effects of fungus, forage, and substrate: liquid ratio were significant ($P < 0.05$) for the laccase activity where the highest laccase activity was recorded in *P. ostreatus* and *T. aestivum* and at 1:5 ratios respectively.

Table 36:The main effect of fungi, forage and substrate-liquid ratio on the laccase activity (µmol / min) after 14days of inoculation

Parameters	**Laccase activity (µmol / min)**
Fungus	
C. rivulosus	64.31
P. ostreatus	76.63
SEM	1.43
Forage	
A. gayanus	44.30^{c}
B. decumbens	9.05^{d}
L. perenne	101.75^{b}
T. aestivum	126.78^{a}
SEM	2.03
Substrate: liquid ratio	
1:3	53.12
1:5	87.82
SEM	1.43

Means with different letters for the same treatment effect are significantly ($P < 0.05$) different, SEM; standard error of means.

The interactive effects of fungus, forage, and substrate-liquid ratio were significant ($P < 0.05$) for the laccase activity (Figure 7). The highest laccase activity produced by *C. rivulosus* was noticed when it was used to inoculate each of the forages at 1: 5 ratio while that of *P. ostreatus* for *B. decumbens*, *A. gayanus* and *L. perenne* was at 1:3 ratio and that of *T. aestivum* was at 1: 5 ratio. The forages varied ($P < 0.05$) in their laccase activity with respect to each fungus. *P ostreatus* at each sub-liquid ratio recorded higher laccase activity in *B. decumbens*, *A. gayanus* and *T. aestivum* than *C. rivulosus* while *C. rivulosus* recorded higher laccase activity in *L. perenne* than *P. ostreatus*

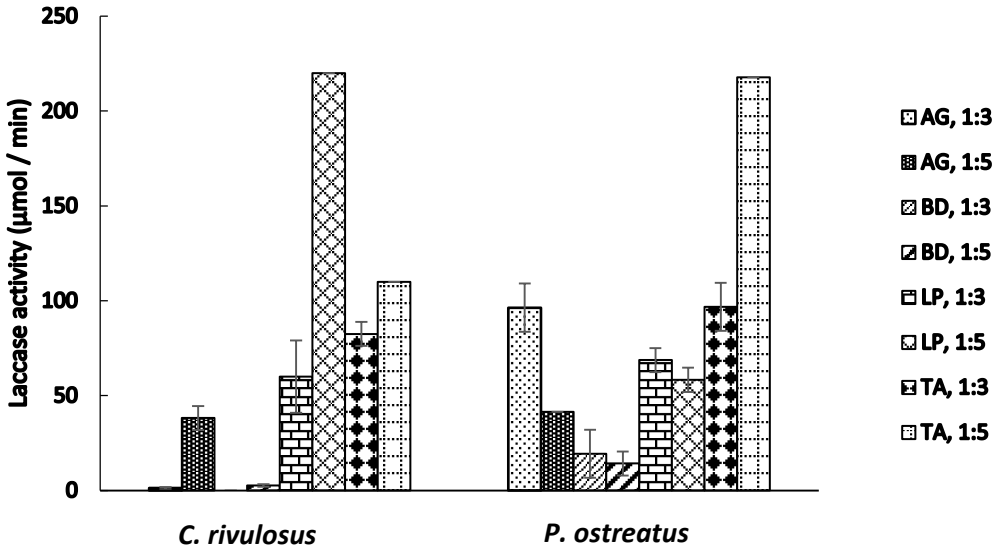


Figure 7 The relationship of fungus, forage and substrate-liquid ratio on the laccase activity (µmol / min) after 14 days of inoculation

The main effects of fungus (*C. rivulosus* and *P. ostreatus*), forage (*A. gayanus*, *B. decumbens*, *L. perenne* and *T. aestivum*) and substrate: liquid ratio (1:3 and 1:5) were significant ($P < 0.05$) for the laccase activity after 28 days of inoculation (Table 37). The highest laccase activities were recorded in *P. ostreatus*, *T. aestivum* and 1: 5 ratios respectively.

Table 37 The main effect of fungus, forage and substrate-liquid ratio on the laccase activity (µmol / min) after 28 days of inoculation

Parameters	**Laccase activity (µmol / min)**
Fungus	
C. rivulosus	86.70
P. ostreatus	117.19
SEM	4.24
Forage	
A. gayanus	72.20^{c}
B. decumbens	61.19^{c}
L. perenne	117.23^{b}
T. aestivum	157.16^{a}
SEM	5.99
Substrate: liquid ratio	
1:3	90.25
1:5	113.64
SEM	4.24

Means with different letters for the same treatment effect are significantly ($P < 0.05$) different, SEM; standard error of means.

The interactive effects of fungus, forage and substrate: liquid ratio produced were significant ($P < 0.05$) for the laccase activity (Figure 8). The highest laccase activity was produced by *C. rivulosus* when it was used to inoculate each of the forages at 1: 5 ratio except in BD, while that of *P. ostreatus* effect on *A. gayanus* and *B. decumbens* was at 1:3 ratio and that of *T. aestivum* and *L. perenne* was at 1: 5 ratio. The forages varied ($P < 0.05$) in their laccase activity with respect to each fungi. *P ostreatus* at each sub-liquid ratio recorded higher laccase activity in *B. decumbens*, *A. gayanus* and *T. aestivum* than *C. rivulosus* while *C. rivulosus* recorded higher laccase activity in *L. perenne* than *P. ostreatus.*

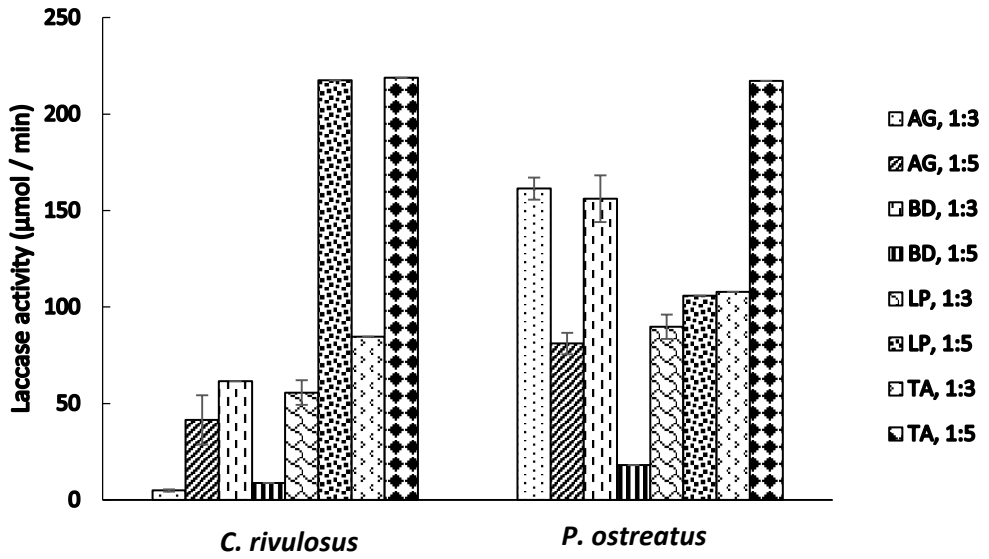
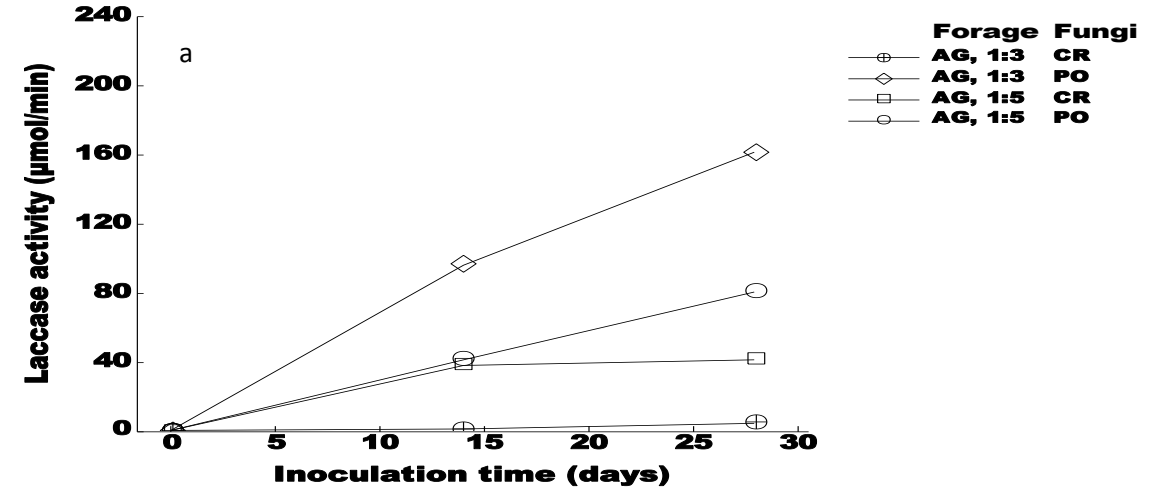
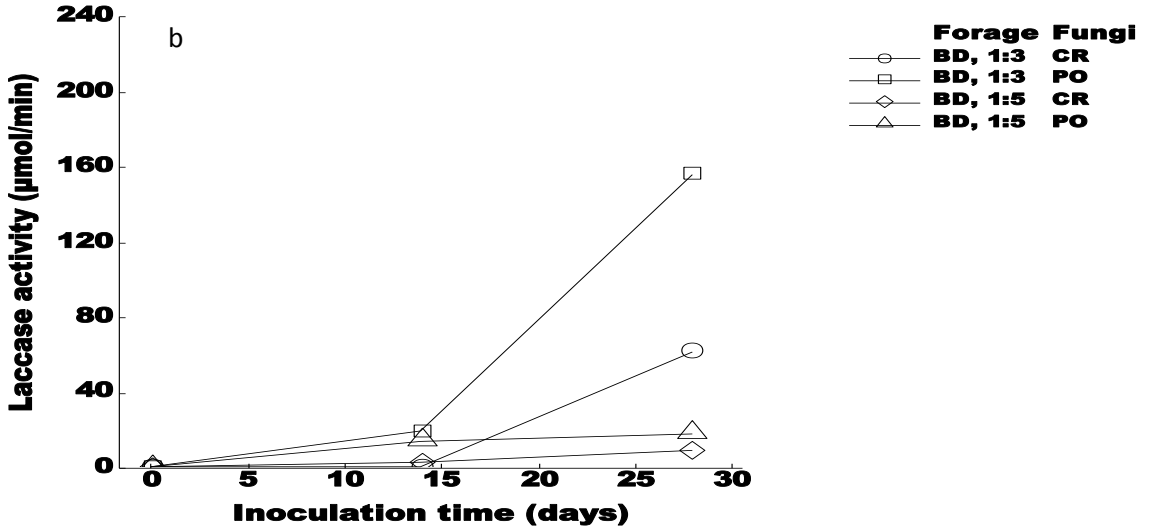
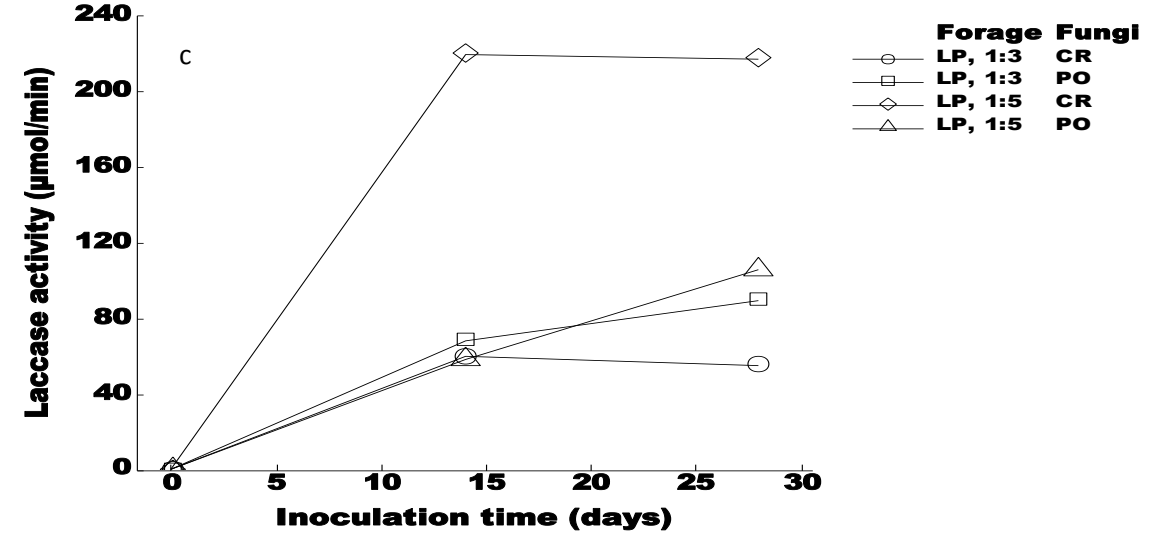


Figure 8 The interactive effects of fungus, forage and substrate-liquid ratio on the laccase activity (µmol / min) after 28 days of inoculation

The laccase activity produced by each of the fungi over the whole duration of the study (28 days of inoculation) is presented in Figure 9. *P. ostreatus* recorded greater laccase production when used to inoculate *A. gayanus*, *B. decumbens*, *T. aestivum* except in *L. perenne* than *C. rivulosus*. In general substrate-liquid ratio 1:3 increased the laccase activity in *A. gayanus* and *B. decumbens* than ratio 1:5 and vice versa in *L. perenne* and *T. aestivum*. Among forages, the highest and the lowest laccase activity were recorded in *T. aestivum* and *B. decumbens*. Among fungi, while considering the substrate-liquid ratio, *C. rivulosus* when inoculated using substrate-liquid ratio 1:5 produced the highest laccase activity. The forages can be grouped into two groups based on their ability to support laccase activity by the fungi: the low laccase producing group (i.e. *B. decumbens* and *A. gayanus*) and the high laccase producing group (i.e. *T. aestivum* and *L. perenne*). The forages also varied in that *L. perenne,* and *T. aestivum* supported a higher production of laccase activity by the fungi by the 14th day of inoculation while for *B. decumbens* and *A. gayanus* it was by the 28 days of inoculation.


a
Laccase activity (µmol/min)
240
200
160
120
80
40
0
0
5
10
15
20
25
30
Inoculation time (days)
Forage Fungi
AG, 1:3 CR
AG, 1:3 PO
AG, 1:5 CR
AG, 1:5 PO


b
Laccase activity (µmol/min)
240
200
160
120
80
40
0
0
5
10
15
20
25
30
Inoculation time (days)
Forage Fungi
BD, 1:3 CR
BD, 1:3 PO
BD, 1:5 CR
BD, 1:5 PO


c
Laccase activity (µmol/min)
240
200
160
120
80
40
0
0
5
10
15
20
25
30
Inoculation time (days)
Forage Fungi
LP, 1:3 CR
LP, 1:3 PO
LP, 1:5 CR
LP, 1:5 PO

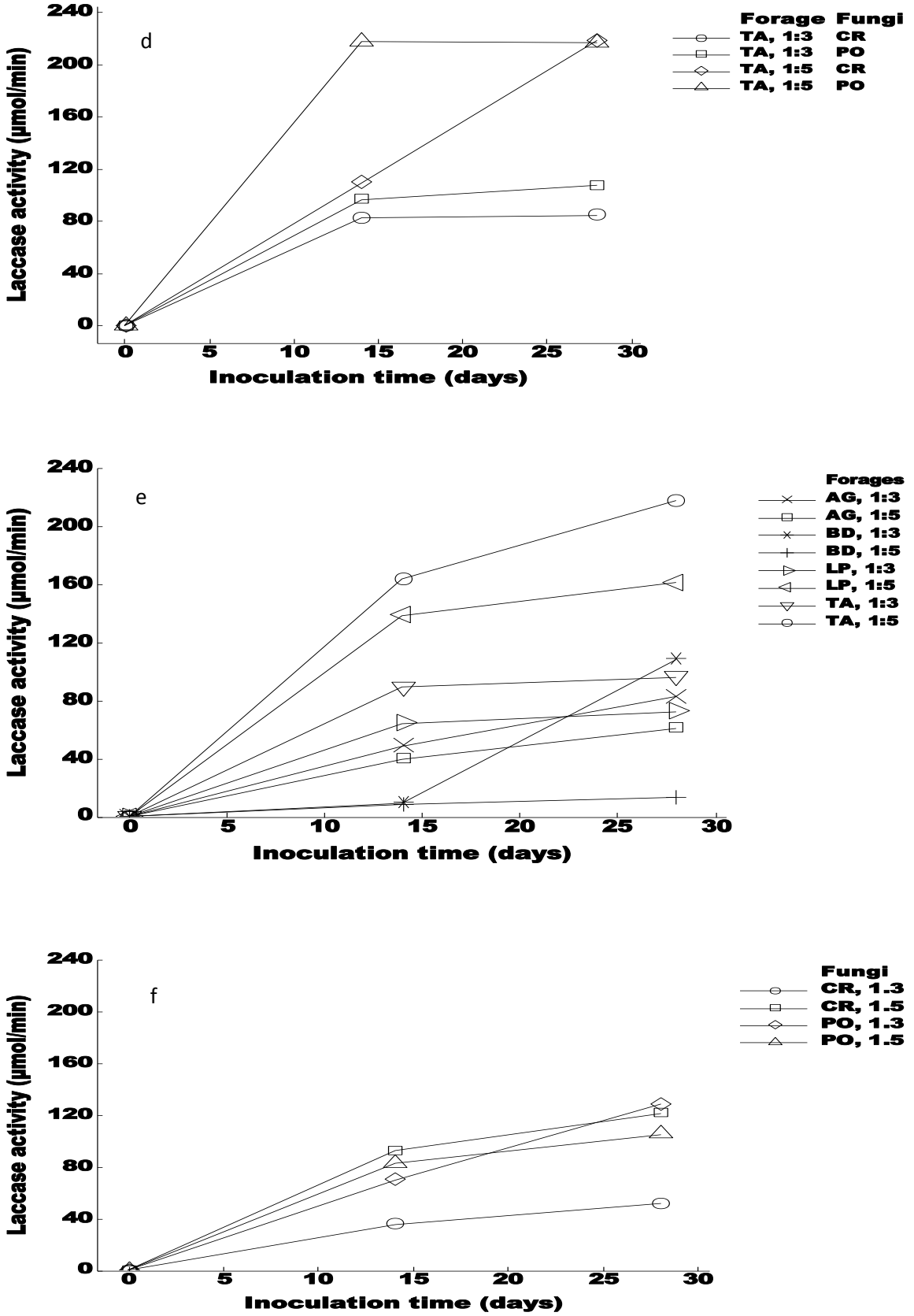


Figure 9 The laccase activity produced by various interactions for 28days a) A. gayanus, substrate-liquid ratio and fungi b) B. decumbens, substrate-liquid ratio and fungi c) L. perenne, substrate-liquid ratio and fungi d) T. aestivum, substrate-liquid ratio and fungi e) Forages and sub-liquid ratios f) Fungi and sub-liquid ratios

5.3.2 *Chemical composition of treated forages as influenced by the filtration method (i.e. PFF and FFF)*

The influence that filtration methods (i.e. PFF and FFF) had on the main effects that fungus and sub-liquid ratio had on the pre-treated forages chemical composition after 14 days of inoculation are presented in Table 38 and Table 39. The filtration method had a significant ($P < 0.05$) influence on the chemical composition of the pre-treated forages. The pre-treated forages obtained from the PFF method recorded lower ash, CP and secondary metabolites contents, as well as higher OM, carbon, fibre and predicted lignin contents than the FFF- treated forages.

The filtration methods influenced the effect that fungal species had on the chemical composition of the pre-treated forages. Pre-treated forages obtained from both filtering methods recorded reduced OM, carbon, and metabolite contents as well as increased ash and CP contents when compared to the untreated forages, with higher reduction and increase in these components being recorded in pre-treated forages obtained from the FFF method. The FFF method led to the reduction in fibre content of the pre-treated forages while the PFF led to an increase in the fibre content of the pre-treated forages when compared to the untreated forages.

The filtration methods influenced the recorded effect of substrate-liquid ratio on the chemical composition of the pre-treated forages. The pre-treated forages at both substrate-liquid ratios obtained from the FFF method recorded lower OM, carbon, fibre (i.e. NDF and ADF), and lignin contents as well as higher metabolites, ash and CP contents than those obtained from the PFF method.

Table 38 The main effects of fungus and substrate- liquid ratio on the chemical composition of 14 days pre-treated forages obtained from PFF method

	Forage				
Parameters	***A. gayanus***	***B. decumbens***	***L perenne***	***T. aestivum***	**SEM**
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	978.66^{bc}	981.80^{ab}	977.70^{c}	983.95^{a}	0.56
Organic matter	967.49^{a}	963.95^{b}	951.31^{d}	958.43^{c}	0.83
Ash	32.51^{d}	36.05^{c}	48.69^{a}	41.57^{b}	0.83
Crude protein	126.28^{b}	61.12^{c}	165.42^{a}	30.39^{d}	0.50
Carbon	459.48^{b}	456.06^{d}	463.88^{a}	457.65^{c}	0.39
NDF	845.35^{c}	859.28^{b}	746.64^{d}	875.37^{a}	0.58
ADF	738.36^{c}	746.24^{b}	635.46^{d}	771.70^{a}	0.61
ADLcal	131.19^{c}	133.08^{b}	106.49^{d}	139.19^{a}	0.12
Hemicellulose	106.99^{b}	113.05^{a}	111.18^{a}	103.67^{c}	0.79
Secondary metabolites (g/kg DM)					
Total phenols	5.58^{a}	4.20^{d}	5.13^{b}	4.42^{c}	0.05
Total tannins	3.02^{a}	1.56^{b}	1.52^{b}	1.69^{b}	0.05
Total antioxidant	4.36^{b}	3.37^{c}	6.07^{a}	3.30^{c}	0.06

	Fungus			
Parameters	**Control**	***C. rivulosus***	***P. ostreatus***	**SEM**
Chemical constituents (g/kg DM)				
Dry matter (g/kg)	985.44^{a}	979.93^{b}	976.29^{c}	0.48
Organic matter	965.49^{a}	960.96^{b}	954.44^{c}	0.72
Ash	34.15^{c}	39.04^{b}	45.56^{a}	0.72
Crude protein	91.53^{a}	98.38^{a}	97.49^{a}	0.43
Carbon	464.60^{a}	457.76^{b}	455.44^{c}	0.34
Neutral Detergent fibre	832.06^{b}	835.06^{a}	827.71^{c}	0.50
Acid detergent fibre	714.79^{b}	727.46^{a}	726.57^{a}	0.53
ADLcal	125.53^{b}	128.57^{a}	128.36^{a}	0.10
Hemicellulose	117.27^{a}	107.75^{b}	101.14^{c}	0.68
Secondary metabolites (g / kg DM)				
Total phenols	6.96^{a}	4.42^{b}	3.11^{c}	0.04
Total tannins	3.18^{a}	1.75^{b}	0.92^{c}	0.04
Total antioxidant	7.11^{a}	3.36^{b}	2.35^{c}	0.05

	Substrate-liquid ratio		
Parameters	1:3	1:5	SEM
Chemical constituents (g/kg DM)			
Dry matter (g/kg)	980.44	980. 96	0.39
Organic matter	960.83	959.76	0.59
Ash	39.17	40.24	0.59
Crude protein	95.33	96.27	0.35
Carbon	457.82	460.72	0.28
Neutral Detergent fibre	831.62	831.70	0.41
Acid detergent fibre	723.48	722.40	0.43
ADLcal	127.61	127.36	0.09
Hemicellulose	108.15	109.29	0.06
Secondary metabolites (g / kg DM)			
Total phenols	5.20^{a}	4.47^{b}	0.03
Total tannins	2.06^{a}	1.84^{b}	0.04
Total antioxidant	4.80^{a}	3.75^{b}	0.04

Means with different letters in the same row are significantly ($P < 0.05$) different, SEM; standard error of means; ADLcal; Acid detergent lignin calculated from regression equation.

Table 39 The main effects of fungus and substrate- liquid ratio on the chemical composition of 14 days pre-treated forages obtained from FFF method.

	Forage				
Parameters	*A. gayanus*	*B. decumbens*	*L. perenne*	*T. aestivum*	SEM
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	988.89^{c}	992.07^{a}	981.93^{d}	990.20^{b}	0.29
Organic matter	951.07^{b}	950.53^{b}	930.36^{c}	958.00^{a}	0.43
Ash	48.93^{b}	49.47^{b}	69.64^{a}	42.00^{c}	0.43
Crude protein	148.56^{b}	67.48^{c}	160.83^{a}	38.05^{d}	0.36
Carbon	460.42^{a}	452.99^{b}	451.47^{c}	449.33^{d}	0.17
Neutral Detergent Fibre	785.93^{c}	817.24^{b}	543.99^{d}	854.05^{a}	0.30
Acid Detergent Fibre	531.28^{c}	542.58^{b}	415.87^{d}	559.92^{a}	0.22
ADLcal	81.49^{c}	84.20^{b}	53.79^{d}	88.36^{a}	0.05
Hemicellulose	254.64^{c}	274.66^{b}	128.11^{d}	294.13^{a}	0.24
Secondary metabolites (g/kg DM)					
Total phenols	6.99^{b}	5.90^{c}	7.32^{a}	5.18^{d}	0.07
Total tannins	3.72^{a}	2.63^{b}	2.68^{b}	2.73^{b}	0.06
Total antioxidant	5.92^{b}	6.15^{b}	7.83^{a}	3.55^{c}	0.07

	Fungus			
Fungi	Control	*C. rivulosus*	*P. ostreatus*	SEM
Chemical constituents (g/kg DM)				
Dry matter (g/kg)	993.40^{a}	986.09^{b}	985.32^{b}	0.25
Organic matter	960.49^{a}	943.75^{b}	938.23^{c}	0.38
Ash	39.51^{c}	56.25^{b}	61.77^{a}	0.38
Crude protein	97.42^{c}	105.33^{b}	108.44^{a}	0.31
Carbon	463.64^{a}	452.08^{b}	444.93^{c}	0.15
Neutral Detergent Fibre	767.16^{a}	745.19^{b}	738.55^{c}	0.26
Acid Detergent Fibre	522.66^{a}	510.78^{b}	503.80^{c}	0.19
ADLcal	79.42^{a}	76.57^{b}	74.89^{c}	0.05
Hemicellulose	244.51^{a}	234.41^{b}	234.74^{c}	0.20
Secondary metabolites (g/kg DM)				
Total phenols	9.33^{a}	6.01^{b}	3.70^{c}	0.06
Total tannins	4.42^{a}	2.75^{b}	1.64^{c}	0.05
Total antioxidant	10.92^{a}	4.46^{b}	2.19^{c}	0.06

	Substrate-liquid ratio		
Sub: Liquid ratio	01:03	01:05	SEM
Chemical constituents (g/kg DM)			
Dry matter (g/kg)	987.25	989.28	0.21
Organic matter	946.63	948.36	0.31
Ash	53.37	51.65	0.31
Crude protein	106.56	100.9	0.26
Carbon	454.45	452.65	0.12
Neutral Detergent Fibre	750.93	749.67	0.21
Acid Detergent Fibre	512.87	511.96	0.15
ADLcal	77.07	76.85	0.04
Hemicellulose	238.06	237.71	0.17
Secondary metabolites (g/kg DM)			
Total phenols	6.95	5.74	0.05
Total tannins	3.11	2.76	0.04
Total antioxidant	6.38	5.34	0.05

Means with different letters in the same row are significantly ($P < 0.05$) different, SEM; standard error of means, DM: dry matter; ADLcal; Acid detergent lignin calculated from regression equation

The influence the filtration methods (i.e. PFF and FFF) had on the interactive effects that fungus and substrate: liquid ratio had on the proximate contents (DM, OM, Ash, carbon, and CP) are recorded in Tables 40 and 41. The filtration methods significantly ($P < 0.05$) influenced the chemical composition of the pre-treated forages at both substrate-liquid ratios. Both filtration methods at each substrate-liquid ratio reduced the DM and OM contents and increased the Ash and CP, with greater reduction and increase in these components being recorded in pre-treated forages obtained from the FFF method. The filtration methods had much influence on *P.ostreatus* pre-treated forages, as they recorded a more significant reduction in OM and carbon as well as a higher increase in CP and OM contents at both substrate-liquid ratios than in *C. rivulosus* pre-treated ones, except in *L. perenne*. The CP content of *C. rivulosus* treated *L. Perenne* and *T. aestivum* in most cases was greater than the P. *ostreatus* treated ones, even when *P. ostreatus* was found to give more reduction in the OM and carbon contents of these substrates.

The filtration methods influenced the substrate-liquid ratio effect on the chemical composition of the pre-treated forages. The substrate-liquid ratio of 1:3 (PFF) and the substrate-liquid ratio of 1:5 (FFF) recorded greater reduction in OM with a corresponding increase in the ash content as well as greater increase in CP contents of most of the pre-treated forages except in *L. perenne*.

Table 40 The interactive effects of fungus and substrate-liquid ratio on the proximate composition (g/kg DM) of 14 days pre-treated forages obtained from PFF method

		Proximate composition (g/kg DM)				
Forage	**Fungus**	**DM (g/kg)**	**OM**	**Ash**	**CP**	**Carbon**
Substrate: liquid ratio 1:3						
A. gayanus	Control	982.14^{bcdef}	973.74^{a}	26.26^{j}	120.59^{g}	463.78^{abcd}
	C. rivulosus	979.58^{defgh}	972.30^{ab}	27.70^{ij}	128.56^{ef}	459.68^{defgh}
	P. ostreatus	972.09^{i}	956.62^{defghi}	43.38^{bcdefg}	131.16^{e}	453.05^{i}
B.decumbens	Control	985.82^{abcd}	969.42^{abc}	30.58^{hij}	61.18^{hi}	459.72^{defgh}
	C. rivulosus	980.59^{defg}	966.09^{abcd}	33.91^{ghij}	63.10^{h}	456.26^{ghi}
	P. ostreatus	976.52^{fghi}	948.78^{hij}	51.23^{abc}	62.72^{h}	440.80^{j}
L.perenne	Control	981.20^{def}	959.69^{cdefg}	40.31^{defgh}	159.24^{d}	466.57^{ab}
	C. rivulosus	979.26^{defghi}	954.26^{efghij}	45.74^{abcdef}	165.94^{bc}	464.12^{abcd}
	P. ostreatus	973.04^{hi}	943.98^{j}	56.02^{a}	162.51^{cd}	464.52^{abcd}
T.aestivum	Control	990.78^{a}	967.44^{abcd}	32.56^{ghij}	29.02^{j}	467.51^{a}
	C. rivulosus	981.63^{cdef}	957.69^{defgh}	42.31^{cdefg}	30.56^{j}	444.87^{j}
	P. ostreatus	978.98^{defghi}	959.94^{cdefg}	40.06^{defgh}	29.37^{j}	452.97^{i}
Substrate: liquid ratio 1:5						
A.gayanus	Control	984.04^{abcde}	972.88^{ab}	27.13^{ij}	121.47^{g}	461.77^{bcdef}
	C. rivulosus	977.93^{efghi}	967.39^{abcd}	32.61^{ghij}	124.63^{fg}	460.87^{cdefg}
	P. ostreatus	976.16^{fghi}	962.03^{bcdef}	37.98^{efghi}	131.26^{e}	457.71^{efghi}
B.decumbens	Control	988.81^{abc}	969.81^{abc}	30.19^{hij}	55.62^{i}	464.40^{abcd}
	C. rivulosus	980.16^{defgh}	966.74^{abcd}	33.26^{ghij}	62.81^{h}	458.45^{efgh}
	P. ostreatus	979.44^{defgh}	962.84^{abcde}	37.16^{fghij}	61.26^{hi}	456.74^{fghi}
L.perenne	Control	981.52^{cdef}	952.92^{efghij}	47.08^{abcdef}	156.65^{d}	467.54^{a}
	C. rivulosus	977.41^{efghi}	945.85ij	54.16^{ab}	178.41^{a}	465.11^{abc}
	P. ostreatus	973.78^{ghi}	951.19^{fghij}	48.82^{abcde}	169.76^{b}	455.44^{hi}
T.aestivum	Control	989.17^{ab}	958.03^{defgh}	41.97^{cdefg}	28.43^{j}	465.54^{abc}
	C. rivulosus	982.85^{bcdef}	957.37^{defgh}	42.63^{cdefg}	33.07^{j}	452.72^{i}
	P. ostreatus	980.30^{defgh}	950.13^{ghij}	49.87^{abcd}	31.88^{j}	462.31^{bcde}
SEM		1.36	2.04	2.04	1.22	0.96

Means with different letters in the same column for each component are significantly ($P < 0.05$) different, SEM; standard error of means.

Table 41 The interactive effect of fungus and substrate-liquid ratio on the proximate composition (g/kg DM) of 14 days pre-treated forages obtained from FFF method

		Proximate composition (g/kg DM)				
Forage	**Fungus**	**DM (g/kg)**	**OM**	**Ash**	**CP**	**Carbon**
Substrate: liquid ratio 1:3						
A.gayanus	Control	991.94^{cd}	964.47^{ab}	35.53^{jk}	141.14^{g}	470.29^{a}
	C. rivulosus	985.49^{ghi}	945.12^{gh}	54.88^{de}	151.57^{ef}	462.16^{c}
	P. ostreatus	986.90^{efgh}	942.46^{h}	57.54^{d}	163.04^{bc}	452.18^{f}
B.decumbens	Control	994.69^{abc}	956.20^{d}	43.80^{h}	67.23^{j}	461.05^{cd}
	C. rivulosus	984.37^{hi}	955.32^{de}	44.68^{gh}	70.79^{ij}	459.94^{cde}
	P. ostreatus	992.51^{bcd}	943.56^{h}	56.45^{d}	73.99^{i}	449.77^{gh}
L.perenne	Control	986.36^{fghi}	950.00^{efg}	50.00^{efg}	159.26^{cd}	462.00^{c}
	C. rivulosus	984.90^{hi}	913.63^{k}	86.37^{a}	159.60^{cd}	441.16^{ij}
	P. ostreatus	971.30^{k}	922.35^{j}	77.66^{b}	167.30^{ab}	448.63^{h}
T.aestivum	Control	996.07^{ab}	962.16^{bc}	37.84^{ij}	38.28^{m}	458.10^{e}
	C. rivulosus	989.64^{def}	954.72^{def}	45.28^{fgh}	43.26^{l}	448.55^{h}
	P. ostreatus	982.90^{ij}	946.60^{fg}	50.40^{ef}	43.24^{l}	439.57^{jk}
Substrate: liquid ratio 1:5						
A.gayanus	Control	996.89^{a}	966.70^{ab}	33.30^{jk}	132.29^{h}	466.53^{b}
	C. rivulosus	986.40^{efghi}	943.84^{h}	56.16^{d}	147.95^{f}	459.60^{de}
	P. ostreatus	985.70^{ghi}	943.86^{h}	56.14^{d}	155.35^{de}	451.76^{fg}
B.decumbens	Control	997.87^{a}	958.42^{cd}	41.58^{hi}	60.13^{k}	462.08^{c}
	C. rivulosus	992.82^{bcd}	953.23^{def}	46.77^{fgh}	62.01^{k}	448.27^{h}
	P. ostreatus	990.14^{de}	936.48^{i}	63.52^{c}	70.70^{ij}	436.81^{l}
L. perenne	Control	985.38^{hi}	957.47^{cd}	42.53^{hi}	151.59^{ef}	470.17^{a}
	C. rivulosus	979.76^{j}	925.72^{j}	74.28^{b}	168.41^{a}	448.84^{h}
	P. ostreatus	983.87^{hi}	913.01^{k}	86.99^{a}	158.81^{cd}	438.02^{kl}
T.aestivum	Control	997.97^{a}	968.53^{a}	31.47^{k}	29.44^{h}	458.83^{de}
	C. rivulosus	989.25^{defg}	958.43^{cd}	41.57^{hi}	39.01^{lm}	448.17^{h}
	P. ostreatus	985.35^{hi}	954.57^{def}	45.43^{fgh}	35.06^{m}	442.76^{i}
SEM		0.72	1.06	1.06	0.89	0.42

Means with different letters in the same column for each ratio are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein.

The influence the filtration methods (i.e. PFF and FFF) produced on the interactive affect that fungus and substrate: liquid ratio had on the fibre (NDF and ADF), and lignin (i.e. ADLcal) contents of pre-treated and untreated forages are recorded in Tables 42 and 43. The filtration methods significantly ($P < 0.05$) influenced the fibre and lignin composition of the pre-treated and untreated forages at both substrate-liquid ratios. The PFF method influence on the pre-treated forages at each of the substrate-liquid ratio led to an increase in the fibre and lignin contents when compared to the untreated forages, except in *P. ostreatus* treated *A. gayanus* and *L. perenne*., whereas the use of the FFF method led to a reduction in the fibre and lignin contents of the pre-treated forages at each substrate-liquid ratio.

The filtration methods had a greater influence on most of the *P.ostreatus* pre-treated forages, as they recorded greater reduction in fibre and lignin contents at both substrate-liquid ratios than *C. rivulosus*, except in *T. aestivum* (PFF) and *L. perenne* (FFF).

The filtration methods influenced the substrate-liquid ratio influence on the fibre and lignin contents of the pre-treated forages. The substrate-liquid ratio of 1:5 recorded greater reduction in fibre and lignin contents of most of the pre-treated forages, except in *A. gayanus* and *B. decumbens* (PFF) and *B. decumbens* (FFF).

Table 42 The interactive effects of fungus and substrate-liquid ratio on the fibre and lignin composition (g/kg DM) of 14 days pre-treated forages obtained from the PFF method

			Fibre fractions (g/kg DM)			
	Forage	**Fungus**	**NDF**	**ADF**	**ADLcal**	**Hemicellulose**
Substrate: liquid ratio 1:3						
	A. gayanus	Control	839.41^{i}	728.84^{m}	128.90^{i}	110.57^{cdef}
		C. rivulosus	854.40^{fgh}	744.99^{ghijk}	132.78^{ghijk}	109.41^{def}
		P. ostreatus	835.46^{i}	737.16^{kl}	130.90^{kl}	98.30^{ghi}
	B. decumbens	Control	858.05^{fg}	740.08^{ijkl}	131.60^{ijkl}	117.97^{abcd}
		C. rivulosus	860.12^{efg}	746.30^{fghi}	133.09^{fghi}	113.82^{bcde}
		P. ostreatus	852.90^{gh}	750.23^{efg}	134.04^{efg}	102.67^{fghi}
	L. perenne	Control	757.48^{j}	635.62^{no}	106.53^{no}	121.86^{ab}
		C. rivulosus	747.97^{kl}	641.55^{n}	107.95^{n}	106.42^{efgh}
		P. ostreatus	741.09^{lm}	634.69^{no}	106.31^{no}	106.40^{efgh}
	T. aestivum	Control	868.25^{cd}	754.46^{de}	135.05^{de}	113.79^{bcde}
		C. rivulosus	881.18^{ab}	782.47^{ab}	141.77^{ab}	98.71^{ghi}
		P. ostreatus	883.15^{a}	785.32^{a}	142.46^{a}	97.84^{ghi}
Substrate: liquid ratio 1:5						
	A. gayanus	Control	848.13^{h}	732.07^{lm}	129.68^{lm}	116.06^{abcde}
		C. rivulosus	856.11^{fg}	741.25^{hijk}	131.88^{hijk}	114.86^{abcde}
		P. ostreatus	838.58^{i}	745.87^{fghij}	132.99^{fghij}	92.71^{lm}
	B. decumbens	Control	858.18^{fg}	738.12^{jkl}	131.13^{jkl}	120.07^{abc}
		C. rivulosus	865.82^{de}	749.26^{efgh}	133.80^{efgh}	116.56^{abcde}
		P. ostreatus	860.64^{ef}	753.44^{def}	134.81^{def}	107.20^{efg}
	L. perenne	Control	755.19^{jk}	630.61^{o}	105.33^{o}	124.58^{a}
		C. rivulosus	740.70^{lm}	640.09^{n}	107.60^{n}	100.60^{fghi}
		P. ostreatus	737.41^{m}	630.21^{o}	105.23^{o}	107.20^{efg}
	T. aestivum	Control	871.81^{cd}	758.51^{d}	136.02^{d}	113.29^{bcde}
		C. rivulosus	875.37^{bc}	773.78^{c}	139.69^{c}	101.60^{fghi}
		P. ostreatus	872.46^{cd}	775.66^{bc}	140.14^{bc}	96.80^{hi}
	SEM		1.41	1.51	0.3	1.94

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means; NDF; neutral detergent fibre, ADF; Acid detergent fibre; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

Table 43 The interactive effect of fungus and substrate-liquid ratio on the fibre and lignin compositions (g/kg DM) of 14 days pre-treated forages obtained from the FFF method.

			Fibre fractions (g/kg DM)			
	Forage	**Fungus**	**NDF**	**ADF**	**ADLcal**	**Hemicellulose**
Substrate: liquid ratio 1:3						
	A. gayanus	Control	801.37^{i}	538.21^{fg}	83.15^{fg}	263.16^{h}
		C. rivulosus	792.01^{j}	532.74^{h}	81.84^{h}	259.28^{i}
		P. ostreatus	763.54^{m}	521.61^{j}	79.17^{j}	241.92^{k}
	B. decumbens	Control	824.19^{fg}	546.66^{de}	85.18^{de}	277.52^{e}
		C. rivulosus	821.77^{a}	545.55^{e}	84.91^{e}	276.22^{e}
		P. ostreatus	799.41^{i}	532.38^{h}	81.75^{h}	267.03^{g}
	L. perenne	Control	567.66^{n}	429.94^{k}	57.17^{k}	137.72^{l}
		C. rivulosus	533.80^{p}	413.47^{l}	53.21^{l}	120.33^{o}
		P. ostreatus	539.90^{o}	409.29^{m}	52.21^{m}	130.60^{n}
	T. aestivum	Control	869.98^{b}	571.98^{a}	91.25^{a}	298.01^{ab}
		C. rivulosus	853.46^{c}	564.08^{b}	89.36^{b}	289.38^{d}
		P. ostreatus	844.09^{d}	548.54^{d}	85.63^{d}	295.55^{bc}
Substrate: liquid ratio 1:5						
	A. gayanus	Control	805.69^{h}	540.57^{f}	83.72^{f}	265.12^{gh}
		C. rivulosus	779.79^{k}	526.10^{i}	80.24^{i}	253.69^{j}
		P. ostreatus	773.17^{l}	528.48^{i}	80.82^{i}	244.69^{k}
	B. decumbens	Control	827.73^{f}	549.31^{d}	85.82^{d}	278.42^{e}
		C. rivulosus	822.13^{g}	544.20^{e}	84.59^{e}	277.93^{e}
		P. ostreatus	808.22^{h}	537.39^{g}	82.95^{g}	270.84^{f}
	L. perenne	Control	566.71^{n}	430.23^{k}	57.23^{k}	136.48^{lm}
		C. rivulosus	514.20^{q}	404.38^{n}	51.03^{n}	109.81^{p}
		P. ostreatus	541.66^{o}	407.92^{m}	51.88^{m}	133.74^{m}
	T. aestivum	Control	873.98^{a}	574.35^{a}	91.82^{a}	299.63^{a}
		C. rivulosus	844.39^{d}	555.76^{c}	87.36^{c}	288.62^{d}
		P. ostreatus	838.39^{e}	544.81^{e}	84.74^{c}	293.577^{c}
	SEM		0.73	0.53	0.13	0.58

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, NDF; neutral detergent fibre, ADF; Acid detergent fibre; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10)

The influence the filtration methods (i.e. PFF and FFF) had on the interactive effects that fungus and substrate: liquid ratio had on the secondary metabolites contents (TP, TT and TAC) are recorded in Tables 44 and 45. The filtration methods significantly ($P < 0.05$) influenced the metabolite contents of the pre-treated forages at both substrate-liquid ratios. Both filtration methods at each of the substrate-liquid ratios reduced the metabolite contents, with greater reduction in metabolite contents being recorded in pre-treated forages obtained from the PFF method. The filtration methods greatly influenced most of the *P.ostreatus* pre-treated forages, as they recorded greater reduction in metabolites content at both substrate-liquid ratios than *C. rivulosus* pre-treated forages, except in *L. perenne* and T.aestivum (PFF). The filtration methods influenced the substrate-liquid ratio effect on the secondary metabolite contents of the pre-treated forages. The substrate-liquid ratio of 1:3 recorded greater reduction in secondary metabolite contents of most of the pre-treated forages, except in *A. gayanus* (PFF) as well as *A. gayanus* and *B. decumbens* (FFF).

Table 44 The interactive effects of fungus and substrate-liquid ratio on the secondary metabolites composition (g/kg DM) of 14 days pre-treated forages obtained from the PFF method

		Secondary metabolites (g/kg DM)		
Forage	**Fungus**	**Total phenols**	**Total tannins**	**Total antioxidant**
Substrate: liquid ratio 1:3				
A. gayanus	Control	9.32^{a}	5.46^{a}	6.92^{b}
	C. rivulosus	5.63^{e}	3.12^{b}	4.20^{de}
	P. ostreatus	3.75^{ij}	1.27^{ghijk}	2.01^{hi}
B. decumbens	Control	5.74^{de}	2.64^{bcd}	4.25^{d}
	C. rivulosus	4.34^{gh}	1.55^{fghi}	3.66^{def}
	P. ostreatus	2.33^{m}	0.51^{l}	1.76^{i}
L. perenne	Control	9.28^{a}	2.86^{bc}	16.78^{a}
	C. rivulosus	5.31^{ef}	1.68^{efgh}	3.81^{def}
	P. ostreatus	4.25^{hi}	1.04^{hijkl}	3.26^{fg}
T. aestivum	Control	6.32^{cd}	2.68^{bcd}	7.43^{b}
	C. rivulosus	2.90^{klm}	1.00^{ijkl}	1.77^{i}
	P. ostreatus	3.15^{kl}	0.94^{ijkl}	1.71^{i}
Substrate: liquid ratio 1:5				
A. gayanus	Control	7.46^{b}	4.99^{a}	7.31^{b}
	C. rivulosus	4.64^{gh}	2.26^{cde}	3.52^{def}
	P. ostreatus	2.65^{lm}	1.05^{hijkl}	2.18^{hi}
B. decumbens	Control	5.30^{ef}	2.13e^{fg}	4.22^{d}
	C. rivulosus	4.88^{fg}	1.88e^{fg}	4.03^{de}
	P. ostreatus	2.61^{lm}	0.64^{kl}	2.30^{hi}
L. perenne	Control	5.72^{e}	1.69^{efgh}	5.82^{c}
	C. rivulosus	3.46j^{k}	1.09^{hijkl}	3.29^{fg}
	P. ostreatus	2.74^{lm}	0.77^{jkl}	3.47^{ef}
T. aestivum	Control	6.49^{c}	2.99^{a}	4.16^{de}
	C. rivulosus	4.22^{hi}	1.42^{ghij}	2.64^{gh}
	P. ostreatus	3.44^{jk}	1.13^{hijkl}	2.11^{hi}
SEM		0.11	0.12	0.14

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means.

Table 45 The interactive effects of fungus and substrate-liquid ratio on the secondary metabolites composition (g/kg DM) of 14 days pre-treated forages obtained from the FFF method

		Secondary metabolites (g/kg DM)		
Forage	**Fungus**	**Total phenols**	**Total tannins**	**Total antioxidant**
Substrate: liquid ratio 1:3				
A. gayanus	Control	11.88^{ab}	7.17^{a}	13.09^{b}
	C. rivulosus	7.51^{de}	3.70^{cde}	5.14^{g}
	P. ostreatus	4.13^{ghi}	2.09^{jklm}	2.05^{j}
B. decumbens	Control	8.04^{cd}	3.96^{cd}	8.09^{e}
	C. rivulosus	7.51^{de}	3.08^{efgh}	8.01^{e}
	P. ostreatus	2.48^{k}	1.01^{n}	1.86^{j}
L. perenne	Control	12.40^{a}	4.44^{c}	18.50^{a}
	C. rivulosus	7.96^{cd}	2.88^{fghi}	5.81^{fg}
	P. ostreatus	5.79^{f}	1.34^{mn}	2.49^{hij}
T. aestivum	Control	7.15^{de}	3.64^{def}	7.65^{e}
	C. rivulosus	4.36^{ghi}	2.05^{klm}	2.08^{j}
	P. ostreatus	4.25^{ghi}	2.02^{lm}	1.77^{j}
Substrate: liquid ratio 1:5				
A. gayanus	Control	11.04^{b}	5.71^{b}	10.81^{c}
	C. rivulosus	4.67^{gh}	2.50^{hijkl}	2.29^{ij}
	P. ostreatus	2.71^{jk}	1.13^{n}	2.12^{j}
B. decumbens	Control	7.91^{cd}	3.55^{defg}	9.36^{d}
	C. rivulosus	6.74^{e}	2.85^{ghij}	7.54^{e}
	P. ostreatus	2.70^{jk}	1.32^{mn}	2.02^{j}
L. perenne	Control	8.65^{c}	3.11^{efgh}	13.74^{b}
	C. rivulosus	5.02^{fg}	2.16^{ijkl}	3.14^{hi}
	P. ostreatus	4.08^{hi}	2.16^{ijkl}	3.28^{h}
T. aestivum	Control	7.56^{de}	3.82^{cde}	6.16^{f}
	C. rivulosus	4.31^{ghi}	2.82^{ghijk}	1.69^{j}
	P. ostreatus	3.47^{ij}	2.04^{m}	1.97^{j}
SEM		0.17	0.15	0.18

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means.

The influence that filtration methods (i.e. PFF and FFF) produced on the effects the interaction of fungus and substrate-liquid ratio showed on the gross nutrient loss of the pre-treated forages after 14 days of inoculation are presented in Tables 46 and 47. The pre-treated forages from the PFF method recorded less loss in OM and carbon contents, as well as less gain in CP and ash, than the pre-treated forages obtained from the FFF method at each substrate-liquid ratio. The PFF method led to increase in the fibre and lignin content of most of the pre-treated forages at each of the substrate-liquid ratios compared to the FFF method, where fibre and lignin loss was recorded in the pre-treated forages at each of the substrate-liquid ratios.

The PFF method led to less TP and TAC contents loss in most of the pre-treated forages in the substrate-liquid ratio of 1:5 than those from the FFF method. On the other hand, when pre-treated forages were in the substrate-liquid ratio of 1:3, they showed different responses to the influence that filtration methods had on their TP loss; lesser TP loss was recorded in *A. gayanus* and *B. Decumbens,* and more loss was recorded in *L. perenne* and *T. aestivum* compared to the pre-treated forages from the FFF method. Regarding the TAC loss in pre-treated forages in the substrate-liquid ratio of 1:3, the fungi varied in their responses. The PFF method led to less loss and higher loss in TAC content of *P. ostreatus* and *C. rivulosus* treated forages respectively than the FFF method treated ones. The PFF method led to more TT loss in most of the pre-treated forages than those obtained from the FFF method at each of the substrate-liquid ratios.

Table 46 The interactive effect of fungus and substrate-liquid ratio on the proximate, fibre and secondary metabolite constituents' loss/gain (%) of 14 days pre-treated forages obtained from the PFF method.

		Proximate contents loss / gain (%)					Fibre content loss / gain (%)			Metabolite contents loss (%)			
Forage	**Fungus**	**DM**	**OM**	**Ash**	**CP**	**Carbon**	**NDF**	**ADF**	**ADLcal**	**Hem**	**TP**	**TT**	**TAC**
Substrate: liquid ratio 1:3													
AG	*C. rivulosus*	0.26^{b}	0.15^{d}	-5.46^{ab}	-6.61^{abcd}	0.88^{fg}	-1.79^{f}	-2.22^{d}	-3.01^{d}	1.05^{d}	39.71^{fg}	42.90^{cde}	38.86^{de}
	P. ostreatus	1.02^{ab}	1.76^{ab}	-65.16^{d}	-8.77^{abcde}	2.32^{cde}	0.47^{cd}	-1.14^{abcd}	-1.55^{abcd}	11.10^{abc}	59.81^{ab}	76.80^{a}	70.40^{a}
BD	*C. rivulosus*	0.53^{ab}	0.34^{cd}	-10.89^{abc}	-3.14^{ab}	0.75^{fg}	-0.24^{de}	-0.84^{abc}	-1.14^{abc}	3.52^{bcd}	24.31^{h}	41.36^{de}	15.21^{f}
	P. ostreatus	0.94^{ab}	2.13^{a}	-67.54^{d}	-2.52^{ab}	4.12^{ab}	0.60^{cd}	-1.37^{cd}	-1.85^{cd}	12.97^{abc}	59.35^{abc}	80.72^{a}	58.81^{b}
LP	*C. rivulosus*	0.20^{b}	0.57^{bcd}	-13.49^{abc}	-4.21^{abc}	0.52^{fg}	1.26^{bc}	-0.93^{abcd}	-1.34^{abcd}	12.68^{abc}	42.85^{efg}	41.38^{de}	76.24^{a}
	P. ostreatus	0.83^{ab}	1.64^{abc}	-38.98^{bcd}	-2.05^{ab}	0.44^{fg}	2.16^{a}	-0.15^{a}	0.21^{a}	12.69^{abc}	54.22^{bcd}	63.79^{abc}	78.90^{a}
TA	*C. rivulosus*	0.92^{ab}	1.01^{abcd}	-29.95^{abc}	-5.32^{abcd}	4.84^{a}	-1.49^{f}	-3.71^{e}	-4.98^{e}	13.26^{abc}	54.08^{bcd}	62.75^{abcd}	76.48^{a}
	P. ostreatus	1.19^{a}	0.78^{bcd}	-23.04^{abc}	-1.21^{a}	3.11^{bc}	-1.72^{f}	-4.09^{e}	-5.48^{e}	14.02^{a}	50.20^{cde}	64.85^{ab}	76.93^{a}
Substrate: liquid ratio 1:5													
AG	*C. rivulosus*	0.62^{ab}	0.56^{bcd}	-20.22^{abc}	-2.60^{ab}	0.19^{g}	-0.94^{ef}	-1.26^{bcd}	-1.70^{bcd}	1.03^{d}	37.82^{fg}	54.77^{bcde}	52.47^{bc}
	P. ostreatus	0.80^{ab}	1.12^{abcd}	-40.00^{cd}	-8.06^{abcde}	0.88^{fg}	1.13^{bc}	-1.89^{cd}	-2.55^{cd}	20.12^{a}	64.46^{a}	79.04^{a}	69.73^{a}
BD	*C. rivulosus*	0.88^{ab}	0.32^{cd}	-10.18^{abc}	-12.92^{de}	1.28^{efg}	-0.89^{ef}	-1.51^{cd}	-2.04^{cd}	2.92^{cd}	7.99^{i}	11.71^{f}	3.06^{g}
	P. ostreatus	0.95^{ab}	0.72^{bcd}	-23.10^{abc}	-10.13^{bcde}	1.65^{def}	-0.29^{de}	-2.08^{cd}	-2.80^{cd}	10.72^{abc}	50.88^{bcde}	70.00^{ab}	44.33^{cde}
LP	*C. rivulosus*	0.42^{ab}	0.74^{bcd}	-15.02^{abc}	-13.89^{de}	0.52^{fg}	1.92^{ab}	-1.50^{cd}	-2.16^{cd}	19.25^{a}	39.59^{fg}	35.31^{e}	40.73^{de}
	P. ostreatus	0.79^{ab}	0.18^{d}	-3.68^{a}	-8.38^{abcde}	2.59^{cd}	2.35^{a}	0.06^{ab}	0.09^{ab}	13.95^{ab}	52.20^{bcde}	54.60^{bcde}	39.59^{de}
TA	*C. rivulosus*	0.64^{ab}	0.07^{d}	-1.58^{a}	-16.30^{e}	2.76^{cd}	-0.41^{de}	-2.01^{cd}	-2.69^{cd}	10.32^{abc}	34.92^{a}	52.44^{bcde}	34.37^{e}
	P. ostreatus	0.90^{ab}	0.82^{abcd}	-18.81^{abc}	-12.12^{cde}	0.70^{fg}	-0.08^{de}	-2.26^{d}	-3.03^{d}	14.56^{a}	46.97^{def}	62.23^{abcd}	46.66^{cd}
SEM		0.17	0.26	6.76	1.69	0.25	0.21	0.21	0.3	0.4	1.86	4.29	2.04

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent fibre; ADF; Acid detergent fibre, TP; total phenols, TT; total tannins, TA; total antioxidant; AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*. ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

Table 47 The interactive effects of fungus and substrate-liquid ratio on the chemical composition and secondary metabolite constituents' loss/gain (%) of 14 days pre-treated forages obtained from the FFF method.

		Proximate contents loss / gain (%)					Fibre content loss / gain (%)			Metabolite contents loss (%)			
Forage	**Fungus**	**DM**	**OM**	**Ash**	**CP**	**Carbon**	**NDF**	**ADF**	**ADLcal**	**Hem**	**TP**	**TT**	**TAC**
Substrate: liquid ratio 1:3													
AG	*C. rivulosus*	0.65^{efg}	2.01^{ef}	-54.44^{ef}	-7.40^{abcd}	1.73^{u}	1.17^{h}	1.02^{g}	1.58^{h}	1.48^{ghi}	36.73^{e}	48.48^{def}	60.69^{h}
	P. ostreatus	0.51^{fgh}	2.28^{de}	-61.95^{fg}	-15.52^{ef}	3.85^{d}	47.21^{c}	3.08^{e}	4.79^{f}	8.07^{c}	65.26^{bc}	70.91^{abc}	84.32^{ab}
BD	*C. rivulosus*	1.04^{bcde}	0.09^{i}	-2.01^{a}	-5.30^{abc}	0.24^{k}	0.29^{i}	0.21^{h}	0.32^{i}	0.47^{hi}	6.63^{f}	22.13^{h}	1.07^{j}
	P. ostreatus	0.22^{gh}	1.32^{g}	-28.86^{cd}	-10.05^{bcde}	2.45^{gh}	3.01^{e}	2.61^{ef}	4.02^{fg}	3.78^{de}	69.11^{ab}	74.58^{ab}	77.03^{cde}
LP	*C. rivulosus*	0.15^{h}	3.83^{b}	-72.74^{g}	-0.22^{a}	4.51^{c}	5.96^{b}	3.83^{cd}	6.92^{de}	12.62^{b}	35.81^{e}	35.19^{fgh}	68.62^{fg}
	P. ostreatus	1.53^{a}	2.91^{cd}	-55.31^{ef}	-5.05^{abc}	2.90^{fg}	4.89^{c}	4.80^{b}	8.67^{bc}	5.17^{d}	53.35^{d}	69.70^{abc}	86.57^{a}
TA	*C. rivulosus*	0.65^{efg}	0.77^{gh}	-19.66^{bc}	-13.03^{def}	2.09^{hi}	1.90^{g}	1.38^{g}	2.08^{h}	2.90^{efg}	39.03^{e}	43.62^{efg}	72.82^{def}
	P. ostreatus	1.32^{ab}	1.31^{g}	-33.18^{cd}	-12.97^{def}	4.05^{cd}	2.98^{ef}	4.10^{c}	6.16^{e}	0.83^{hi}	40.57^{e}	44.55^{efg}	76.80^{cde}
Substrate: liquid ratio 1:5													
AG	*C. rivulosus*	1.05^{bcde}	2.37^{de}	-68.65^{fg}	-11.84^{cdef}	1.49^{j}	3.22^{e}	2.68^{ef}	4.15^{fg}	4.31^{de}	57.71^{cd}	56.18^{cde}	78.86^{c}
	P. ostreatus	1.12^{abcd}	2.36^{de}	-68.60^{fg}	-17.43^{ef}	3.17^{ef}	4.04^{d}	2.24^{f}	3.47^{g}	7.70^{e}	75.51^{a}	80.25^{a}	80.43^{bc}
BD	*C. rivulosus*	0.51^{fgh}	0.54^{hi}	-12.49^{ab}	-3.13^{ab}	2.99^{efg}	0.68^{hi}	0.93^{g}	1.43^{h}	0.17^{i}	14.75^{f}	19.55^{h}	19.45^{i}
	P. ostreatus	0.78^{def}	2.29^{de}	-52.78^{ef}	-17.58^{ef}	5.47^{b}	2.36^{fg}	2.17^{f}	3.34^{g}	2.72^{efg}	65.81^{be}	62.87^{bcd}	78.41^{c}
LP	*C. rivulosus*	0.57^{fgh}	3.32^{bc}	-74.64^{g}	-11.09^{cde}	4.54^{c}	9.27^{a}	6.01^{a}	10.84^{a}	19.54^{a}	41.95^{e}	30.74^{gh}	77.12^{cd}
	P. ostreatus	0.15^{h}	4.64^{a}	-104.53^{h}	-4.76^{abc}	6.84^{a}	4.42^{cd}	5.19^{b}	9.36^{b}	2.01^{fgh}	52.82^{d}	30.74^{gh}	76.16^{cde}
TA	*C. rivulosus*	1.27^{abc}	1.04^{gh}	-32.10^{cd}	-32.53^{g}	2.32^{h}	3.39^{e}	3.24^{de}	4.86^{f}	3.67^{def}	43.00^{e}	26.30^{h}	72.57^{ef}
	P. ostreatus	0.87^{cdef}	1.44^{fg}	-44.35^{de}	-19.13^{f}	3.50^{de}	4.07^{d}	5.14^{b}	7.72^{cd}	2.02^{fgh}	54.09^{d}	46.72^{defg}	67.94^{g}
SEM		0.09	0.13	3.24	1.51	0.11	0.13	0.13	0.21	0.35	1.85	3.32	0.89

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent, ADF; Acid detergent fibre, TP; total phenols, TT; total tannins, TA; total antioxidant, AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*. ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

The influence that filtration methods (i.e. PFF and FFF) produced on the main effects that fungus and substrate-liquid ratio had on the pre-treated forages chemical composition after 28 days of inoculation are presented in Tables 48 and Table 49. The filtration method had a great influence on the chemical composition of the pre-treated forages, with the forages also responding differently to the control. The PFF method lowered the OM and increased the ash content of pre-treated *T. aestivum* and *B. decumbens*, while it increased the OM content and decreased the ash content of pre-treated *A. gayanus* and *L. perenne* in comparison with the FFF method. The pre-treated forages from the PFF method recorded lower CP and metabolite contents as well as higher carbon, fibre, and lignin contents than those from the FFF method.

The filtration methods influenced the recorded effect of fungal species on the chemical composition of the pre-treated forages. Both filtration methods showed that fungal treatment reduced the OM, carbon, and metabolites (i.e. TP, TT, and TAC) contents, as well as increased the ash and CP contents of the pre-treated forages compared to the untreated forages. A higher reduction and increase in these components was recorded in pre-treated forages obtained from the FFF method, except in the TAC content. The FFF method showed that fungal treatment reduced fibre and lignin contents of the pre-treated forages. The PFF method used showed that fungal treatment reduced NDF in all pre-treated forages; treatment reduced ADF in *P. ostreatus* treated forages but not in *C. rivulosus* pre-treated ones, and increased ADLcal in all pre-treated forages when compared to the untreated forages.

The pre-treated forages at both substrate-liquid ratios obtained from the FFF method recorded lower OM, carbon, fibre (i.e. NDF and ADF), and lignin contents as well as higher metabolites, ash and CP contents than those obtained from the PFF method.

Table 48 The main effects of fungus and substrate- liquid ratio on the chemical composition of 28 days pre-treated forages obtained from the PFF method.

		Forage			
Parameters	***A. gayanus***	***B. decumbens***	***L perenne***	***T. aestivum***	**SEM**
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	983.81^{b}	988.87^{a}	985.62^{ab}	987.69^{a}	0.46
Organic matter	947.49^{a}	942.02^{ab}	941.59^{ab}	938.67^{b}	0.76
Ash	52.51^{b}	57.99^{ab}	58.42^{ab}	61.33^{a}	0.76
Crude protein	134.58^{b}	63.07^{c}	175.47^{a}	33.82^{d}	0.89
Carbon	453.60^{a}	450.03^{b}	454.23^{a}	449.84^{b}	0.53
Neutral Detergent fibre	834.23^{c}	851.37^{b}	744.53^{d}	868.40^{a}	0.52
Acid detergent fibre	731.03^{c}	740.27^{b}	634.82^{d}	767.40^{a}	0.78
ADLcal	129.43^{c}	131.64^{b}	106.34^{d}	138.16^{a}	0.15
Hemicellulose	103.20^{b}	111.10^{a}	109.72^{a}	101.00^{b}	0.94
Secondary metabolites (g/kg DM)					
Total phenols	7.18^{a}	5.21^{d}	6.07^{b}	5.62^{c}	0.08
Total tannins	3.18^{a}	1.92^{c}	2.79^{b}	2.66^{b}	0.05
Total antioxidant	5.37^{a}	4.22^{b}	4.28^{b}	3.88^{c}	0.03
		Fungus			
Parameters	**Control**	***C. rivulosus***	***P. ostreatus***	**SEM**	
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	990.65^{a}	985.39^{b}	983.45^{b}	0.4	
Organic matter	950.31^{a}	944.86^{b}	932.16^{c}	0.66	
Ash	49.69^{c}	55.15^{b}	67.84^{a}	0.66	
Crude protein	91.18^{a}	107.63^{a}	106.39^{a}	0.77	
Carbon	462.627^{a}	450.57^{b}	442.59^{c}	0.46	
Neutral Detergent fibre	833.46^{a}	823.42^{b}	817.01^{c}	0.45	
Acid detergent fibre	714.89^{b}	72.20^{a}	718.04^{b}	0.68	
ADLcal	125.55^{c}	127.31^{a}	126.31^{b}	0.13	
Hemicellulose	118.57^{a}	101.22^{b}	98.97^{b}	0.81	
Secondary metabolites (g / kg DM)					
Total phenols	7.96^{a}	5.75^{b}	4.35^{c}	0.07	
Total tannins	3.66^{a}	2.53^{b}	1.72^{c}	0.05	
Total antioxidant	6.61^{a}	3.94^{b}	2.76^{c}	0.03	
	Substrate-liquid ratio				
Parameters	1:3	1:5	SEM		
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	985.28	987.72	0.33		
Organic matter	942.61	942.28	0.54		
Ash	57.39	57.73	0.54		
Crude protein	103.41	100.06	0.63		
Carbon	452.83	451.03	0.37		
Neutral Detergent fibre	825.07	824.19	0.37		
Acid detergent fibre	719.35	717.40	0.56		
ADLcal	126.63	126.16	0.11		
Hemicellulose	105.72	106.78	0.66		
Secondary metabolites (g / kg DM)					
Total phenols	6.13	5.91	0.05		
Total tannins	2.69	2.58	0.04		
Total antioxidant	4.45	4.43	0.02		

Means with different letters in the same column for each factor effect are significantly ($P < 0.05$) different, SEM; standard error of mean; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

Table 49 The main effects of forage, fungus and substrate-liquid ratio on the chemical and secondary metabolite compositions (g/kg DM) of 28 days pre-treated forages obtained from the FFF method.

	Forage				
Parameters	*A. gayanus*	*B. decumbens*	*L. perenne*	*T. aestivum*	SEM
Chemical constituents (g/kg DM)					
DM (g/kg)	977.33^{b}	980.24^{a}	975.13^{c}	980.14^{a}	0.46
Organic matter	942.64^{b}	947.60^{b}	923.88^{c}	952.15^{a}	0.56
Ash	57.36^{b}	52.40^{c}	76.13^{a}	47.85^{d}	0.56
Crude protein	151.29^{b}	69.59^{c}	173.75^{a}	36.42^{d}	0.43
Carbon	463.42^{a}	447.98^{b}	448.51^{b}	440.50^{c}	0.25
Neutral Detergent Fibre	768.47^{c}	798.59^{b}	523.84^{d}	849.84^{d}	0.35
Acid Detergent Fibre	516.84^{c}	521.24^{b}	400.74^{d}	552.37^{a}	0.36
ADLcal	78.02^{c}	80.52^{b}	50.16^{d}	86.55^{a}	0.09
Hemicellulose	251.63^{c}	271.35^{b}	123.10^{d}	297.51^{a}	0.33
Secondary metabolites (g/kg DM)					
Total phenols	10.01^{a}	7.17^{c}	8.91^{b}	6.51^{d}	0.09
Total tannins	5.27^{a}	3.04^{c}	3.89^{b}	2.76^{d}	0.08
Total antioxidant	5.97^{b}	5.30^{c}	7.17^{a}	3.79^{d}	0.04
	Fungus				
Parameters	Control	*C. rivulosus*	*P. ostreatus*	SEM	
Chemical constituents (g/kg DM)					
DM (g/kg)	988.98^{a}	976.61^{b}	969.03^{c}	0.40	
Organic matter	961.17^{a}	936.45^{b}	927.08^{c}	0.49	
Ash	38.83^{c}	63.55^{b}	72.92^{a}	0.49	
Crude protein	94.59^{c}	111.29^{b}	117.40^{a}	0.37	
Carbon	468.46^{a}	445.08^{b}	436.78^{c}	0.21	
Neutral Detergent Fibre	768.07^{a}	727.24^{b}	710.27^{c}	0.30	
Acid Detergent Fibre	520.44^{a}	495.76^{b}	481.69^{c}	0.31	
ADLcal	78.89^{a}	72.96^{b}	69.59^{c}	0.07	
Hemicellulose	247.64^{a}	231.48^{b}	228.58^{c}	0.28	
Secondary metabolites (g/kg DM)					
Total phenols	12.301^{a}	6.44^{b}	5.71^{c}	0.08	
Total tannins	6.581^{a}	2.66^{b}	1.97^{c}	0.07	
Total antioxidant	10.818^{a}	3.34^{b}	2.51^{c}	0.03	
	Substrate-liquid ratio				
Parameters	1:3	1:5	SEM		
Chemical constituents (g/kg DM)					
DM (g/kg)	980.61	975.81	0.32		
Organic matter	940.36	942.77	0.40		
Ash	59.64	57.23	0.40		
Crude protein	108.01	107.52	0.30		
Carbon	450.58	449.63	0.18		
Neutral Detergent Fibre	735.26	735.13	0.25		
Acid Detergent Fibre	500.09	498.5	0.25		
ADLcal	74.00	73.62	0.06		
Hemicellulose	235.17^{b}	236.63^{a}	0.23		
Secondary metabolites (g/kg DM)					
Total phenols	8.48	7.82	0.06		
Total tannins	3.93	3.54	0.05		
Total antioxidant	5.80	5.31	0.03		

Means with different letters in the same column for each factor effect are significantly ($P < 0.05$) different, SEM; standard error of means; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

The influence the filtration methods (i.e. PFF and FFF) produced on the interactive affects that fungus and substrate: liquid ratio had on the proximate contents (DM, OM, Ash, carbon, and CP) of pre-treated forages is recorded in Tables 50 and 51. The filtration methods significantly ($P < 0.05$) influenced the interactive effects that fungus and substrate-liquid ratio had on the chemical composition of the pre-treated forages. Both filtration methods at each substrate-liquid ratio reduced the DM and OM contents and increased the Ash and CP, with more reduction and increase in these components being recorded in pre-treated forages obtained from the FFF method. The filtration methods greatly influenced *P. ostreatus* pre-treated forages, as they recorded greater reduction in OM and carbon as well as a higher increase in CP and OM contents at both substrate-liquid ratios than *C. rivulosus* pre-treated forages, except in *L. perenne* (PFF) and *T. aestivum* (FFF). In the PFF substrates, the CP content of *C. rivulosus* treated *L. perenne* and *T. aestivum* in most cases was greater than the P. *ostreatus* treated ones, even when *P. ostreatus* was found to have given greaterreduction in OM and carbon contents of these substrates.

The filtration methods influenced the substrate-liquid ratio effect on the chemical composition of the pre-treated forages. The substrate-liquid ratio of 1:5 (PFF) and substrate-liquid ratio of 1:3 (FFF) supported greater reduction in OM which led to the corresponding higher ash content, as well as greater increase in CP contents of most of the pre-treated forages than the substrate-liquid ratio of 1:3, except in *B. decumbens* (PFF) and *L. perenne* (FFF).

Table 50 The interactive effects of fungus and substrate-liquid ratio on the proximate composition (g/kg DM) of 28 days pre-treated forages obtained from the PFF method.

			Proximate composition (g/kg DM)				
	Forage	**Fungus**	**DM (g/kg)**	**OM**	**Ash**	**CP**	**Carbon**
Substrate: liquid ratio 1:3							
	A.gayanus	Control	987.10^{bcde}	957.68^{ab}	42.32^{jk}	120.36^{f}	464.91^{ab}
		C. rivulosus	981.59^{efg}	950.16^{abcd}	49.84^{hijk}	152.11^{cd}	448.05^{hijkl}
		P. ostreatus	981.14^{efg}	935.92^{ghij}	64.09^{bcde}	142.77^{de}	446.17^{jklm}
	B.decumbens	Control	994.05^{a}	949.44^{bcde}	50.56^{ghij}	61.13^{g}	458.25^{bcdef}
		C. rivulosus	985.30^{bcdefg}	943.87^{defgh}	56.14^{defgh}	62.16^{g}	453.26^{fghi}
		P. ostreatus	983.85^{cdefg}	929.56^{jk}	70.44^{ab}	63.52^{g}	436.34^{n}
	L.perenne	Control	991.24^{ab}	950.67^{abcd}	49.33^{hijk}	156.3^{c}	465.99^{a}
		C. rivulosus	979.70^{g}	945.68^{cdefg}	54.32^{efghi}	196.17^{a}	460.91^{abcde}
		P. ostreatus	980.60^{fg}	929.19^{jk}	70.81^{ab}	177.93^{b}	446.42^{ijklm}
	T.aestivum	Control	984.19^{cdefg}	941.41^{defgh}	58.59^{defgh}	29.21^{i}	463.36^{abc}
		C. rivulosus	989.79^{abc}	940.08^{efghi}	59.92^{cdefg}	34.08^{hi}	440.47^{mn}
		P. ostreatus	984.79^{cdefg}	937.66^{fghij}	62.34^{bcdef}	45.16^{h}	449.80^{ghij}
Substrate: liquid ratio 1:5							
	A.gayanus	Control	989.09^{abc}	955.17^{abc}	44.83^{ijk}	119.55^{f}	466.15^{a}
		C. rivulosus	982.67^{defg}	950.63^{abcd}	49.37^{hijk}	134.45^{e}	456.31^{defg}
		P. ostreatus	981.25^{efg}	935.40^{hij}	64.60^{bcd}	138.22^{e}	439.99^{mn}
	B.decumbens	Control	994.44^{a}	946.71^{cdef}	53.29^{fghi}	58.45^{g}	457.95^{cdef}
		C. rivulosus	988.83^{abc}	944.38^{defgh}	55.62^{defgh}	64.79^{g}	454.20^{efgh}
		P. ostreatus	986.78^{bcde}	938.14^{fghij}	61.86^{bcdef}	68.34^{g}	440.21^{mn}
	L.perenne	Control	991.07^{ab}	959.70^{a}	40.30^{k}	156.80^{c}	461.92^{abcd}
		C. rivulosus	988.41^{abcd}	943.37^{defgh}	56.63^{defgh}	183.06^{b}	448.80^{hijk}
		P. ostreatus	982.69^{defg}	920.90^{k}	79.10^{a}	182.56^{b}	441.37^{lmn}
	T.aestivum	Control	994.06^{a}	941.73^{defgh}	58.27^{defgh}	27.64^{i}	462.49^{abcd}
		C. rivulosus	986.84^{bcde}	940.67^{defgh}	59.33^{defgh}	34.20^{hi}	442.53^{klmn}
		P. ostreatus	986.49^{bcdef}	930.49^{ijk}	69.51^{abc}	32.66^{i}	440.41^{mn}
	SEM		1.13	1.87	1.87	2.18	1.30

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein.

Table 51 The interactive effect of forage, fungus and substrate-liquid ratio on the proximate composition (g/kg DM) of 28 days pre-treated forages obtained from the FFF method.

			Proximate composition (g/kg DM)				
	Forage	**Fungus**	**DM (g/kg)**	**OM**	**Ash**	**CP**	**Carbon**
Substrate: liquid ratio 1:3							
	A.gayanus	Control	986.83^{bcd}	966.62^{ab}	33.38^{lm}	118.44^{e}	478.30^{a}
		C. rivulosus	980.74^{efgh}	933.14^{ij}	66.86^{de}	159.96^{d}	465.36^{d}
		P. ostreatus	980.50^{efgh}	919.72^{k}	80.28^{c}	180.71^{b}	449.77^{f}
	B.decumbens	Control	988.66^{bc}	960.66^{bcd}	39.34^{jkl}	58.16^{i}	464.27^{d}
		C. rivulosus	977.82^{ghij}	949.89^{efg}	50.11^{ghi}	71.59^{fgh}	457.04^{e}
		P. ostreatus	975.91^{hijk}	937.17^{hi}	62.83^{ef}	76.53^{f}	423.91^{lm}
	L.perenne	Control	983.90^{cdef}	950.91^{ef}	49.09^{hi}	169.61^{c}	474.74^{b}
		C. rivulosus	982.26^{defg}	912.63^{kl}	87.37^{bc}	175.62^{b}	439.80^{hi}
		P. ostreatus	962.29^{o}	908.37^{l}	91.63^{b}	176.82^{b}	425.49^{l}
	T.aestivum	Control	991.35^{ab}	963.35^{abc}	36.65^{klm}	32.15^{l}	458.77^{e}
		C. rivulosus	986.21^{bcde}	938.21^{hi}	61.79^{ef}	41.19^{j}	431.86^{k}
		P. ostreatus	964.58^{no}	943.65^{gh}	56.35^{fg}	35.31^{kl}	437.65^{ij}
Substrate: liquid ratio 1:5							
	A.gayanus	Control	989.60^{abc}	968.48^{a}	31.52^{m}	121.89^{e}	478.58^{a}
		C. rivulosus	962.83^{no}	938.02^{hi}	61.98^{ef}	159.69^{d}	459.22^{e}
		P. ostreatus	963.48^{no}	929.86^{j}	70.14^{d}	167.04^{c}	449.31^{f}
	B.decumbens	Control	995.24^{a}	956.94^{cde}	43.06^{ijk}	66.57^{h}	464.71d
		C. rivulosus	972.40^{ijkl}	951.63^{ef}	48.37^{hi}	69.74^{gh}	442.21^{gh}
		P. ostreatus	971.41^{klm}	929.31^{j}	70.69^{d}	74.96^{fg}	435.76^{j}
	L.perenne	Control	981.48^{defgh}	953.69^{def}	46.31^{hij}	156.01^{d}	470.82^{c}
		C. rivulosus	972.33^{jkl}	916.99^{k}	83.02^{c}	177.41^{b}	443.98^{g}
		P. ostreatus	968.53^{lmn}	900.67^{m}	99.33^{a}	187.05^{a}	436.20^{j}
	T.aestivum	Control	994.81^{a}	968.70^{a}	31.30^{m}	33.93^{l}	457.47^{e}
		C. rivulosus	978.30^{fghi}	951.10^{ef}	48.90^{hi}	35.14^{l}	421.14^{m}
		P. ostreatus	965.59^{mno}	947.90^{fg}	52.10^{gh}	40.78^{jk}	436.11^{j}
	SEM		1.12	1.37	1.37	1.05	0.61

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of mean; DM, dry matter; OM, organic matter; CP, crude protein.

The influence the filtration methods (i.e. PFF and FFF) produced on the interactive effects that fungus and substrate: liquid ratio had on the fibre (NDF and ADF) and lignin (i.e. ADLcal) contents of pre-treated forages are recorded in Tables 52 and 53. The filtration methods significantly ($P < 0.05$) influenced the fibre and lignin composition of the pre-treated forages at both substrate-liquid ratios. The PFF method influenced the fibre and lignin contents at each substrate-liquid ratio, in that it reduced the NDF content of most pre-treated forages except *T. aestivum*; it reduced ADF content of *P. ostreatus* treated forages and not *C. rivulosus* treated ones; and increased the ADLcal content of most of the pre-treated forages except in *P. ostreatus* treated *A. gayanus* and *L perenne*. On the other hand, the FFF method influenced the substrate-liquid ratios positively in that fibre and lignin contents were reduced in all the pre-treated forages.

The filtration methods greatly influenced *P. ostreatus* pre-treated forages, as they recorded greater reduction in fibre and lignin contents at both substrate-liquid ratios than *C. rivulosus* pre-treated forages, except in *T. aestivum* (PFF) and *L. perenne* and *T. aestivum* (FFF).

The filtration methods influenced the effect that substrate-liquid ratio had on the fibre and lignin contents of the pre-treated forages. In the PFF and FFF substrates, the substrate-liquid ratio of 1:5 recorded greater reduction in fibre and lignin contents for most of the pre-treated forages, except in *A. gayanus* and *B. decumbens* (PFF) and in *B. decumbens* (FFF).

Table 52 The interactive effect of fungus and substrate-liquid ratio on the fibre composition (g/kg DM) of 28 days pre-treated forages obtained from the PFF method.

			Fibre fractions (g/kg DM)			
	Forage	**Fungus**	**NDF**	**ADF**	**ADLcal**	**Hemicellulose**
Substrate: liquid ratio 1:3						
	A. gayanus	Control	842.02^{def}	730.83^{ghi}	129.38^{ghi}	111.19^{bcdef}
		C. rivulosus	835.58^{f}	734.41^{fghi}	130.24^{fghi}	101.17^{fghijk}
		P. ostreatus	820.48^{g}	728.34^{hi}	128.78^{hi}	92.14^{jk}
	B. decumbens	Control	860.22^{c}	738.33^{efgh}	131.18^{efgh}	121.88^{abc}
		C. rivulosus	848.54^{d}	740.50^{efg}	131.70^{efg}	108.05^{defgh}
		P. ostreatus	836.63^{f}	738.32^{efgh}	131.18^{efgh}	98.31^{ghijk}
	L. perenne	Control	760.43^{h}	637.60^{j}	107.00^{j}	122.83^{ab}
		C. rivulosus	740.99^{i}	640.93^{j}	107.80^{j}	100.05^{fghijk}
		P. ostreatus	737.21^{ij}	633.38^{jk}	105.99^{jk}	103.83^{efghij}
	T. aestivum	Control	871.71^{ab}	756.78^{c}	135.61^{c}	114.93^{abcde}
		C. rivulosus	870.52^{ab}	774.22^{ab}	139.79^{ab}	96.30^{hijk}
		P. ostreatus	876.54^{a}	778.59^{a}	140.84^{a}	97.95^{ghijk}
Substrate: liquid ratio 1:5						
	A. gayanus	Control	844.39^{de}	728.06^{hi}	128.72^{hi}	116.33^{abcd}
		C. rivulosus	839.58^{ef}	739.40^{efg}	131.44^{efg}	100.18^{fghijk}
		P. ostreatus	823.33^{g}	725.16^{i}	128.02^{i}	98.17^{ghijk}
	B. decumbens	Control	859.36^{c}	737.33^{efgh}	130.94^{efgh}	122.036^{abc}
		C. rivulosus	855.38^{c}	745.36^{de}	132.87^{de}	110.02^{cdefg}
		P. ostreatus	848.06^{d}	741.76^{ef}	132.00^{ef}	106.30^{defgh}
	L. perenne	Control	761.03^{h}	635.47^{jk}	106.49^{jk}	125.55^{a}
		C. rivulosus	735.40^{ij}	635.09^{jk}	106.40^{jk}	100.31^{fghijk}
		P. ostreatus	732.12^{j}	626.41^{k}	104.32^{k}	105.71^{defghi}
	T. aestivum	Control	868.52^{b}	754.74^{cd}	135.12^{cd}	113.78^{abcde}
		C. rivulosus	861.36^{c}	767.72^{b}	138.23^{b}	93.65^{ijk}
		P. ostreatus	861.72^{c}	772.35^{ab}	139.34^{ab}	89.37^{jk}
	SEM		1.27	1.92	0.38	2.29

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

Table 53 The interactive effect of fungus and substrate-liquid ratio on the fibre and secondary metabolites composition (g/kg DM) of 28 days pre-treated forages obtained from the FFF method.

		Fibre fractions (g/kg DM)			
Forage	**Fungus**	**NDF**	**ADF**	**ADLcal**	**Hemicellulose**
Substrate: liquid ratio 1:3					
A. gayanus	Control	803.93^{f}	536.97^{e}	82.85^{e}	266.96^{h}
	C. rivulosus	779.07^{h}	524.39^{fg}	79.83^{fg}	254.68^{i}
	P. ostreatus	718.07^{k}	487.24^{k}	70.92^{k}	230.82^{l}
B. decumbens	Control	828.52^{d}	546.87^{bc}	85.23^{bc}	281.65^{e}
	C. rivulosus	790.28^{g}	521.16^{gh}	79.06^{gh}	269.12^{gh}
	P. ostreatus	747.05^{j}	501.70^{j}	74.39^{j}	245.35^{jk}
L. perenne	Control	562.05^{l}	427.70^{l}	56.63^{l}	134.36^{m}
	C. rivulosus	522.18^{m}	409.11^{m}	52.17^{m}	113.08^{p}
	P. ostreatus	505.53^{n}	383.27^{n}	45.96^{n}	122.26^{o}
T. aestivum	Control	875.62^{a}	567.99^{a}	90.30^{a}	307.64^{a}
	C. rivulosus	850.04^{b}	549.70^{b}	85.91^{b}	300.34^{b}
	P. ostreatus	840.73^{c}	544.98^{cd}	84.78^{cd}	295.75^{l}
Substrate: liquid ratio 1:5					
A. gayanus	Control	808.65^{e}	539.54^{e}	83.47^{e}	269.11^{gh}
	C. rivulosus	757.43^{i}	510.56^{i}	76.52^{j}	246.87^{j}
	P. ostreatus	743.70^{j}	502.35^{i}	74.54^{j}	241.35^{k}
B. decumbens	Control	829.09^{d}	547.13^{bc}	85.29^{bc}	281.96^{e}
	C. rivulosus	804.95^{ef}	527.98^{f}	80.70^{f}	276.96^{f}
	P. ostreatus	791.63^{g}	518.57^{h}	78.44^{h}	273.06^{fg}
L. perenne	Control	562.63^{l}	425.17^{l}	56.02^{l}	137.46^{m}
	C. rivulosus	486.68^{o}	384.78^{n}	46.33^{n}	101.90^{q}
	P. ostreatus	503.97^{n}	374.44^{o}	43.85^{o}	129.53^{n}
T. aestivum	Control	874.09^{a}	572.13^{a}	91.29^{a}	301.96^{b}
	C. rivulosus	827.29^{d}	538.41^{e}	83.20^{e}	288.88^{d}
	P. ostreatus	831.51^{d}	540.98^{de}	83.82^{de}	290.52^{d}
SEM		0.86	0.87	0.21	0.8

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means; ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

The influence the filtration methods (i.e. PFF and FFF) produced on the interactive effects that fungus and substrate: liquid ratio had on the secondary metabolites content (TP, TT and TAC) are recorded in Tables 54 and 55. The filtration methods significantly ($P < 0.05$) influenced the metabolite contents of the pre-treated forages at both substrate-liquid ratios. Both filtration methods at each substrate-liquid ratio reduced the metabolite contents, with more reduction in TP and TT contents being recorded in most of the pre-treated forages obtained from the PFF method than those from the FFF method. The TAC contents of most of the pre-treated forages in the substrate-liquid ratio of 1:3 obtained from the PFF method were greater than the FFF substrates, but vice versa was recorded with pre-treated forages in the substrate-liquid ratio of 1:5.

The filtration methods greatly influenced most of the *P. ostreatus* pre-treated forages, as they recorded greater reduction in metabolites content at both substrate-liquid ratios in comparison with *C. rivulosus* pre-treated forages, except in *L. perenne* (PFF) as well as *L. perenne* and *T. aestivum* (FFF). The filtration methods influenced the effect that the substrate-liquid ratio had on the secondary metabolite contents of the pre-treated forages. In the substrate-liquid ratio of 1:5 a greater reduction in secondary metabolites content was recorded with *A. gayanus* and *L. perenne*, but not with *B. decumbens* and *T. aestivum* using both PFF and FFF methods.

Table 54 The interactive effects of fungus and substrate-liquid ratio on the secondary metabolites composition (g/kg DM) of 28 days pre-treated forages obtained from the PFF method.

		Secondary metabolites (g/kg DM)		
Forage	**Fungus**	**Total phenols**	**Total tannins**	**Total antioxidant**
Substrate: liquid ratio 1:3				
A. gayanus	Control	10.10^{ab}	5.15^{a}	7.97^{b}
	C. rivulosus	8.24^{d}	3.34^{bcd}	5.60^{d}
	P. ostreatus	4.30^{jk}	1.38^{ij}	2.74^{gh}
B. decumbens	Control	5.94^{f}	2.29^{fgh}	5.43^{d}
	C. rivulosus	4.93^{ghij}	1.89^{ghij}	4.01^{f}
	P. ostreatus	4.15^{jk}	1.50^{ij}	2.90^{g}
L. perenne	Control	9.31^{bc}	3.84^{b}	8.21^{b}
	C. rivulosus	5.72^{fg}	3.07^{cde}	3.02^{g}
	P. ostreatus	4.54^{ijk}	2.28^{fgh}	2.19^{i}
T. aestivum	Control	6.15^{ef}	3.09^{cde}	4.71^{e}
	C. rivulosus	5.56^{fgh}	2.84^{def}	3.81^{f}
	P. ostreatus	4.58^{ijk}	1.66^{hij}	2.82^{gh}
Substrate: liquid ratio 1:5				
A. gayanus	Control	10.79^{a}	5.48^{a}	8.97^{a}
	C. rivulosus	5.48^{fghi}	2.34^{fgh}	3.87^{f}
	P. ostreatus	4.18^{jk}	1.36^{j}	3.03^{g}
B. decumbens	Control	5.95^{f}	2.26^{fgh}	5.31^{d}
	C. rivulosus	6.37^{ef}	2.09^{ghi}	4.81^{e}
	P. ostreatus	3.92^{k}	1.49^{ij}	2.87^{g}
L. perenne	Control	8.52^{cd}	3.70^{bc}	7.50^{c}
	C. rivulosus	3.92^{k}	2.08^{ghi}	2.28^{i}
	P. ostreatus	4.39^{jk}	1.79^{hij}	2.50^{hi}
T. aestivum	Control	6.93^{e}	3.51^{bcd}	4.81^{e}
	C. rivulosus	5.78^{fg}	2.59^{efg}	4.12^{f}
	P. ostreatus	4.73^{hijk}	2.27^{fgh}	3.04^{g}
SEM		0.18	0.13	0.07

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means

Table 55 The interactive effect of fungus and substrate-liquid ratio on the secondary metabolites composition (g/kg DM) of 28 days pre-treated forages obtained from the FFF method.

		Secondary metabolites (g/kg DM)		
Forage	**Fungus**	**Total phenols**	**Total tannins**	**Total antioxidant**
Substrate: liquid ratio 1:3				
A. gayanus	Control	18.70^{a}	11.80^{a}	12.24^{c}
	C. rivulosus	8.26^{gh}	4.12^{ef}	5.44^{h}
	P. ostreatus	5.20^{klm}	1.58^{kl}	2.30^{lmn}
B. decumbens	Control	10.66^{de}	5.15^{d}	10.91^{d}
	C. rivulosus	5.91^{jkl}	2.32^{ijkl}	3.15^{ij}
	P. ostreatus	5.04^{klm}	1.52^{l}	2.28^{lmn}
L. perenne	Control	12.88^{c}	6.88^{c}	16.64^{a}
	C. rivulosus	9.21^{fg}	3.67^{fgh}	3.33^{i}
	P. ostreatus	6.86^{ij}	2.14^{ijkl}	2.41^{klmn}
T. aestivum	Control	9.72^{ef}	4.76^{de}	6.97^{f}
	C. rivulosus	4.46^{m}	1.52^{l}	2.00^{mn}
	P. ostreatus	4.88^{m}	1.76^{jkl}	1.98^{n}
Substrate: liquid ratio 1:5				
A. gayanus	Control	16.51^{b}	9.88^{b}	10.95^{d}
	C. rivulosus	6.10^{jk}	2.52^{ijk}	2.41^{klmn}
	P. ostreatus	5.30^{klm}	1.70^{kl}	2.46^{klm}
B. decumbens	Control	9.94^{def}	4.86^{de}	7.50^{e}
	C. rivulosus	6.59^{ij}	2.73^{hij}	5.24^{h}
	P. ostreatus	4.90^{lm}	1.65^{1kl}	2.73^{jkl}
L. perenne	Control	10.99^{d}	5.38^{d}	14.91^{b}
	C. rivulosus	5.82^{jkl}	2.17ijkl	2.31^{lmn}
	P. ostreatus	7.68^{hi}	3.07^{ghi}	3.40^{i}
T. aestivum	Control	9.01^{fg}	3.95^{efg}	6.43^{g}
	C. rivulosus	5.17^{klm}	2.20^{ijkl}	2.79^{jk}
	P. ostreatus	5.81^{jkl}	2.35^{ijkl}	2.55^{kl}
SEM		0.21	0.19	0.09

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means

The influence the filtration methods (i.e. PFF and FFF) produced on the effects that the interaction of fungi and substrate-liquid ratio showed on the gross nutrient loss of the pre-treated forages after 28 days of inoculation is presented in Tables 56 and 57. The pre-treated forages from the PFF method recorded less loss in OM, carbon, fibre and lignin contents, as well as less gain in CP and ash, compared to the pre-treated forages obtained from the FFF method at each substrate-liquid ratio. However, the PFF method led to ADF gain in fungal pre-treated *B. decumbens* and *T. aestivum*, and *C. rivulosus* treated *A. gayanus* and *L. perenne*, at both substrate-liquid ratios. The pre-treated forages from the PFF method recorded less TP loss, except in *L. perenne*, TT loss and TA loss than those obtained from the FFF method at both substrate-liquid ratios. The PFF and FFF methods influenced the substrate-liquid ratio, where the substrate-liquid ratio of 1:3 led to a higher nutrient loss in pre-treated *A. gayanus* and *B. decumbens*, but not in the pre-treated *L. perenne* and *T. aestivum*

Table 56 The interactive effects of fungus and substrate-liquid ratio on the chemical composition and secondary metabolite constituents' loss (+) / gain (-) % of 28 days pre-treated forages obtained from the PFF method.

		Proximate contents loss / gain (%)				Fibre contents loss/ gain (%)				Metabolites loss/gain (%)			
Forage	**Fungus**	**DM**	**OM**	**Ash**	**CP**	**Carbon**	**NDF**	**ADF**	**ADLcal**	**Hem**	**TP**	**TT**	**TAC**
Substrate: liquid ratio 1:3													
AG	*C. rivulosus*	0.56^{de}	0.79^{de}	-17.78^{abc}	-26.38^{f}	3.63^{bcd}	0.76^{def}	-0.49^{bcde}	-0.66^{bcde}	9.01^{b}	24.43^{f}	18.41^{gh}	35.10^{ef}
	P. ostreatus	0.60^{cde}	2.27^{b}	-51.44^{d}	-18.62^{cdef}	4.03^{abc}	2.56^{bc}	0.34^{abc}	0.46^{abc}	17.14^{ab}	66.35^{abc}	57.48^{ab}	73.13^{a}
BD	*C. rivulosus*	0.88^{abcd}	0.59^{de}	-11.02^{a}	-3.91^{ab}	1.09^{e}	1.36^{d}	-0.29^{bcde}	-0.40^{bcde}	11.35^{ab}	25.20^{f}	17.12^{h}	17.58^{gh}
	P. ostreatus	1.03^{abc}	2.09^{bc}	-39.32^{bcd}	-1.69^{a}	4.78^{ab}	2.74^{bc}	0.00^{abcd}	0.00^{abcd}	19.34^{ab}	46.55^{d}	30.20^{ef}	34.69^{ef}
LP	*C. rivulosus*	1.16^{a}	0.52^{de}	-10.11^{a}	-25.51^{ef}	1.09^{e}	2.56^{bc}	-0.52^{bcde}	-4.03^{i}	18.54^{ab}	60.69^{bc}	38.54^{d}	20.00^{g}
	P. ostreatus	1.07^{ab}	2.26^{b}	-43.54^{d}	-13.84^{abcde}	4.20^{abc}	3.05^{abc}	0.66^{ab}	0.75^{bcde}	15.47^{ab}	72.40a	51.26^{bc}	40.68^{de}
TA	*C. rivulosus*	-0.57^{g}	0.14^{e}	-2.27^{a}	16.68^{cdef}	4.94^{ab}	0.14^{fg}	-2.31^{fg}	-3.09^{fg}	16.21^{ab}	17.55fg	9.52^{i}	8.01^{h}
	P. ostreatus	-0.06^{f}	0.40^{e}	-6.40^{a}	-18.62^{cdef}	2.93^{cd}	-0.56^{g}	-2.88^{g}	-3.86^{g}	14.78^{ab}	39.46^{de}	25.51^{fg}	46.32^{cd}
Substrate: liquid ratio 1:5													
AG	*C. rivulosus*	0.65^{bcde}	0.48^{e}	-10.14^{a}	-12.47^{abcd}	2.11^{de}	0.57^{def}	-1.56^{defg}	-2.12^{defg}	13.89^{ab}	56.55^{c}	49.19^{c}	57.24^{b}
	P. ostreatus	0.79^{abcd}	2.07^{bc}	-44.11^{d}	-15.62^{bcdef}	5.61^{a}	2.50^{c}	0.40^{abc}	0.54^{abc}	15.61^{ab}	67.60^{ab}	61.26^{a}	75.15^{a}
BD	*C. rivulosus*	0.56^{de}	0.25^{e}	-4.38^{a}	-10.84^{abc}	0.82^{e}	0.46^{ef}	-1.09^{cdef}	-1.47^{cdef}	9.84^{ab}	8.64^{g}	-7.05^{j}	7.73^{h}
	P. ostreatus	0.77^{abcd}	0.91^{cde}	-16.10^{ab}	-16.92^{cdef}	3.87^{bc}	1.32^{de}	-0.60^{bcde}	-0.81^{bcde}	12.90^{ab}	44.54^{de}	34.07^{de}	34.22^{ef}
LP	*C. rivulosus*	0.27^{ef}	1.70^{bcd}	-40.51^{cd}	-16.74^{cdef}	2.84^{cd}	3.37^{ab}	0.06^{abc}	0.09^{abc}	20.11^{ab}	69.32^{ab}	53.93^{bc}	43.75^{cde}
	P. ostreatus	0.85^{abcd}	4.04^{a}	-96.26^{e}	-16.42^{cdef}	4.45^{abc}	3.80^{a}	1.43^{a}	2.04^{a}	15.80^{ab}	66.20^{abc}	48.49^{c}	51.62^{bc}
TA	*C. rivulosus*	0.73^{abcde}	0.11^{e}	-1.82^{a}	-23.75^{def}	4.32^{abc}	0.82^{def}	-1.72^{efg}	-2.31^{efg}	17.69^{ab}	11.72^{g}	16.65^{h}	26.29^{fg}
	P. ostreatus	0.76^{abcd}	1.19^{bcde}	-19.30^{abc}	-18.19^{cdef}	4.78^{ab}	0.78^{def}	-2.33^{fg}	-3.13^{fg}	21.45^{a}	35.61^{e}	31.75^{def}	35.22^{ef}
SEM		0.09	0.24	4.7	2.41	0.32	0.17	0.31	0.35	2.3	1.98	1.39	2.02

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent, ADF: acid detergent fibre, TP; total phenols, TT; total tannins, TA; total antioxidant, AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*. ADLcal; Acid detergent lignin calculated from regression equation (see figure 10).

Table 57 The interactive effects of fungus and substrate-liquid ratio on the chemical composition and secondary metabolite constituents' loss (+) / gain (-) % of 28 days pre-treated forages obtained from the FFF method.

		Proximate contents loss / gain (%)				Fibre contents loss/ gain (%)				Metabolites loss/gain (%)			
Forage	**Fungus**	**DM**	**OM**	**Ash**	**CP**	**Carbon**	**NDF**	**ADF**	**ADLcal**	**Hem**	**TP**	**TT**	**TAC**
Substrate: liquid ratio 1:3													
AG	*C. rivulosus*	0.62^{ghi}	3.46^{def}	-100.30^{gh}	-35.96^{gh}	2.71^{f}	3.09^{j}	2.34^{l}	3.65^{i}	4.60^{ef}	55.82^{bc}	65.10^{bcd}	55.51^{h}
	P. ostreatus	0.64^{ghi}	4.85^{ab}	-140.51^{j}	-52.58^{i}	5.97^{d}	10.68^{b}	9.26^{cd}	14.40^{c}	13.54^{c}	72.20^{a}	86.57^{a}	81.22^{abc}
BD	*C. rivulosus*	1.10^{efgh}	1.12^{jk}	-27.36^{a}	-23.09^{de}	1.56^{o}	4.62^{hi}	4.70g^{hi}	7.24^{fg}	4.45^{ef}	44.53^{cdef}	54.98^{cdef}	71.10^{f}
	P. ostreatus	1.29^{defg}	2.45ghi	-59.69^{bcd}	-31.59^{fgh}	8.69^{b}	9.83^{c}	8.26^{d}	12.72^{d}	12.89^{c}	52.74^{bcd}	70.52^{abc}	79.09^{bc}
LP	*C. rivulosus*	0.17^{i}	4.03^{bcde}	-77.98^{cdef}	-3.54^{a}	7.36^{c}	7.09^{e}	4.35^{hij}	7.88^{f}	15.84^{b}	28.49^{g}	46.68^{def}	79.97^{abc}
	P. ostreatus	1.56^{def}	4.47^{bc}	-86.65^{efg}	-4.25^{a}	10.37^{a}	10.06^{bc}	10.39^{b}	18.83^{b}	9.00^{d}	46.77^{cde}	68.92^{abc}	85.54^{a}
TA	*C. rivulosus*	0.52^{hi}	2.61^{fghi}	-68.59^{bcde}	-28.12^{ef}	5.87^{d}	2.92^{j}	3.22^{kl}	4.86^{hi}	2.37^{fg}	54.16^{bc}	68.15^{abc}	71.26^{ef}
	P. ostreatus	2.70^{a}	2.05^{hi}	-53.75^{b}	-9.83^{bc}	4.60^{e}	3.99^{i}	4.05^{ijk}	6.12^{gh}	3.86^{efg}	49.82^{cd}	63.06^{bcde}	71.66^{def}
Substrate: liquid ratio 1:5													
AG	*C. rivulosus*	2.71^{a}	3.15^{efg}	-96.61^{fgh}	-31.01^{fg}	4.05^{e}	6.33^{f}	5.39^{fgh}	8.33^{f}	8.26^{d}	63.06^{ab}	74.47^{abc}	77.99^{c}
	P. ostreatus	2.64^{ab}	3.99^{bcde}	-122.49^{ij}	-37.04^{h}	6.12^{d}	8.03^{d}	6.89^{e}	10.70^{e}	10.31^{d}	67.88^{a}	82.83^{ab}	77.57^{cd}
BD	*C. rivulosus*	2.30^{abc}	0.56^{k}	-12.33^{a}	-4.76^{ab}	4.84^{e}	2.91^{j}	3.50^{jk}	5.39^{h}	1.77^{g}	33.70^{fg}	43.76^{ef}	30.17^{i}
	P. ostreatus	2.39^{ab}	2.89^{fgh}	-64.16^{bcd}	-12.60^{c}	6.23^{d}	4.52^{hi}	5.22^{fgh}	8.04^{f}	3.16^{fg}	50.72^{cd}	66.02^{bcd}	63.62^{g}
LP	*C. rivulosus*	0.93^{fgh}	3.85^{cde}	-79.25^{defg}	-13.71^{c}	5.70^{d}	13.50^{a}	9.50^{bc}	17.31^{b}	25.87^{a}	47.04^{cd}	59.63^{cdef}	84.50^{ab}
	P. ostreatus	1.96^{bcd}	5.56^{a}	-114.49^{hi}	-19.90^{d}	7.35^{c}	10.43^{bc}	11.93^{a}	21.73^{a}	5.77^{e}	30.17^{g}	43.05^{ef}	77.18^{cde}
TA	*C. rivulosus*	1.66^{cde}	1.86^{ij}	-56.21^{bc}	-3.57^{a}	7.94^{bc}	5.35^{g}	5.89^{ef}	8.86^{f}	4.33^{ef}	42.59^{def}	44.26^{ef}	56.60^{h}
	P. ostreatus	2.94^{a}	2.15^{hi}	-66.42^{bcde}	-20.20^{d}	4.67^{e}	4.87^{gh}	5.44^{fg}	8.19^{f}	3.79^{efg}	35.57^{efg}	40.28^{f}	60.37^{gh}
SEM		0.14	0.18	4.38	1.10	0.16	0.13	0.21	0.33	0.45	2.26	4.09	1.21

Means with different letters in the same column are significantly ($P < 0.05$) different, SEM; standard error of means, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent, ADF; acid detergent fibre, TP; total phenols, TT; total tannins, TA; total antioxidant, AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*, ADLcal^{a}&^{b}; Acid detergent lignin calculated from regression equation (see figure 10)

5.3.3 *The degradability, fermentation profiles and total gas production of 14 days pre-treated forages obtained from the pump filtered method (PFF)*

The influence that the PFF method produced on the main effects that fungus and incubation time had on the degradability and fermentation parameters of the fourteen days pre-treated forages is presented in Table 58. The pre-treated forages recorded significant ($P < 0.05$) differences in their IVDMD (*in vitro* dry matter degradability) and IVOMD (*in vitro* organic matter degradability), ammonia-nitrogen (NH_3-N; mg/L), methane (CH_4; L / Kg OM), total gas production (tGP; L / kg OM) and pH. The highest digestibility was recorded in pre-treated *L. perenne*, followed by *A. gayanus*, then *B decumbens* and lastly by *T. aestivum*.

The PFF method influenced the effect that fungus had on the fermentation and digestibility parameters of the pre-treated forages, in that it led to a reduction in IVDMD and IVOMD, similar NH_3-N content (mg / L), increased pH, decreased CH_4 poduction and tGP respectively when compared to untreated forages, with *P. ostreatus* pre-treated forages recording more reduction and increase in these components respectively than *C. rivulosus* pre-treated forages. The influence PFF method had on the incubation time was expected, as the parameters measured increased with increase in incubation time.

Table 58 The main effects of fungus and incubation time on the degradability and fermentation parameters of 14 days pre-treated forages obtained from the PFF method

			Forage		
Parameter	***A. gayanus***	***B. decumbens***	***L. perenne***	***T. aestivum***	**SEM**
IVDMD (g/kg DM)	370.79[b]	311.16[c]	611.44[a]	274.84[d]	12.9
IVOMD (g/kg DM)	384.67[b]	330.98[c]	631.54[a]	297.97[d]	12.9
NH_3-N (mg/L)	36.39[a]	35.79[a]	39.18[a]	36.15[a]	1.0
pH	6.77[c]	6.81[b]	6.59[d]	6.84[a]	0.01
CH_4 (L/kg DOM)	23.59[b]	18.62[c]	37.29[a]	16.24[d]	0.8
tGP (L/Kg DOM)	210.43[b]	181.38[c]	330.45[a]	160.97[d]	6.4
% CH4/TGP	11.21	10.27	11.29	10.09	
			Fungus		
Parameter	**Control**	**CR**	**PO**	**SEM**	
IVDMD (g/kg DM)	442.77[a]	395.35[b]	338.05[c]	12.9	
IVOMD (g/kg DM)	461.03[a]	415.09[b]	357.75[c]	12.9	
NH_3-N (mg/L)	38.22[a]	37.10[a]	35.31[a]	1.0	
pH	6.71[c]	6.75[b]	6.80[a]	0.01	
CH_4 (L/kg DOM)	28.20[a]	23.56[b]	20.04[c]	0.8	
tGP (L/Kg DOM)	245.29[a]	222.42[b]	194.71[c]	6.4	
% CH_4/Tgp	11.50	10.59	10.29		
		Incubation time (hrs)			
Parameter	24	48	96	SEM	
IVDMD (g/kg DM)	320.16[c]	403.51[b]	452.50[a]	12.9	
IVOMD (g/kg DM)	339.56[c]	421.55[b]	472.76[a]	12.9	
NH_3-N (mg/L)	27.47[c]	36.69[b]	46.48[a]	1.0	
pH	6.81[a]	6.74[b]	6.70[c]	0.01	
CH_4 (L/kg DOM)	15.00[c]	24.29[b]	32.51[a]	0.8	
tGP (L/Kg DOM)	186.04[c]	228.09[b]	248.30[a]	6.4	
% CH_4/Tgp	8.06	10.65	13.09		

Means with different letters in the same row for each treatment are significantly ($P < 0.05$) different; SEM, standard error of mean. SEM: Standard error of means; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; NH_3-N: ammonia-nitrogen; CH_4: methane; tGP: total gas production; CR: *C. rivulosus*; PO: *P. ostreatus*

Table 59 shows the influence that the PFF method produced on the interactive effects that fungus and incubation time had on the nutrient degradability (IVDMD and IVOMD) and fermentation profiles (NH_3-N, CH_4, and pH) of the pre-treated forages. The influence of the PFF method on the interaction produced a significant effect ($P < 0.05$) on the IVDMD and IVOMD, NH_3-N (mg / L), pH, CH_4 (L/kg DM and OM), and tGP (L /kg OM) of the pre-treated and untreated forages. The IVDMD, IVOMD, NH_3-N, CH_4 content, tGP of the treated and untreated forages increased, while the pH decreased, with increase in incubation time. The PFF method had a negative influence on the ability of the fungi to improve the digestibility and fermentation of the pre-treated forages as it reduced the IVDMD and IVOMD, reduced the NH_3-N content, increased the pH, reduced the CH_4 production and tGP at each inoculation time compared to the untreated forages. The only exception was the higher NH_3-N content recorded in almost all the pre-treated forages, except *L. perenne* at 96h of incubation, than the untreated forages. *P. ostreatus* pre-treated forages recorded lower IVDMD and IVOMD, except in *T. aestivum* at 96h of incubation, lower CH_4 and tGP, lower NH_3-N content and higher pH than *C. rivulosus* pre-treated forages.

Table 59 The interactive effects of fungus and incubation time on the degradability and fermentation parameters of 14days pre-treated forages obtained from the PFF method

For	Fungi	IVDMD (g/kg DM)	IVOMD (g/kg OM)	NH_3-N (mg/l)	Ph	CH_4 (ml/g DOM)	tGP (ml/g DOM)	% CH_4/ TGP
24h of incubation								
AG	Con	304.05^{i}	314.49^{j}	34.19^{abcdef}	6.81^{g}	15.32^{no}	188.19^{ghi}	8.14
	CR	233.76^{lmn}	246.93^{lmn}	26.44^{bcdef}	6.87^{cde}	11.02^{opq}	147.39^{jklmno}	7.48
	PO	215.50^{mn}	229.50^{mn}	24.39^{def}	6.89^{bcd}	9.70^{pq}	136.73^{lmno}	7.09
BD	Con	273.90^{ijkl}	294.20^{jk}	28.91^{abcdef}	6.83^{efg}	13.28^{op}	166.44^{ijklm}	7.98
	CR	263.60^{ijkl}	285.14^{jkl}	27.64^{bcdef}	6.85^{def}	12.37^{op}	160.24^{ijklmno}	7.72
	PO	191.47^{no}	206.80^{no}	21.48^{ef}	6.91^{b}	8.93^{pq}	126.29^{o}	7.07
LP	Con	607.44^{abc}	622.06^{abc}	36.69^{abcdef}	6.61^{lm}	32.04^{cde}	326.87^{a}	9.80
	CR	579.39^{bcd}	597.61^{bcd}	34.10^{abcdef}	6.64^{kl}	26.82^{fghi}	316.98^{ab}	8.46
	PO	573.20^{cd}	593.58^{cd}	27.62^{bcdef}	6.65^{jk}	25.09^{hijk}	314.25^{ab}	7.97
TA	Con	245.83^{jklm}	285.16^{jkl}	25.20^{cdef}	6.85^{efg}	10.67^{opq}	140.26^{klmno}	7.61
	CR	202.95^{mn}	222.42^{mn}	22.63^{ef}	6.89^{bc}	8.58^{pq}	127.63^{no}	6.72
	PO	150.76^{o}	176.86^{o}	20.30^{f}	6.97^{a}	6.19^{q}	81.21^{p}	7.62
48h of incubation								
AG	Con	435.76^{f}	444.53^{fg}	38.85^{abcdef}	6.72^{i}	29.63^{efgh}	250.48^{cde}	11.83
	CR	401.90^{fgh}	419.45^{fghi}	37.96^{abcdef}	6.75^{hi}	24.21^{ijkl}	228.55^{def}	10.59
	PO	293.21^{i}	299.39^{jk}	34.97^{abcdef}	6.82^{fg}	18.88^{mn}	183.64^{ghij}	10.28
BD	Con	403.30^{fgh}	423.07^{fgh}	37.54^{abcdef}	6.74^{hi}	25.10^{hijk}	226.80^{ef}	11.07
	CR	364.87^{h}	381.41^{i}	36.82^{abcdef}	6.77^{h}	20.66^{klm}	208.05^{fgh}	9.93
	PO	210.20^{mn}	229.12^{mn}	31.68^{abcdef}	6.89^{bcd}	11.34^{op}	130.87^{mno}	8.67
LP	Con	639.13^{a}	660.94^{a}	39.79^{abcdef}	6.55^{o}	39.42^{b}	337.62^{a}	11.68
	CR	602.77^{abc}	626.85^{abc}	38.25^{abcdef}	6.60^{lmn}	35.48^{bcd}	324.22^{a}	10.94
	PO	613.01^{abc}	634.25^{ab}	38.73^{abcdef}	6.59^{mn}	36.63^{bc}	333.40^{a}	10.99
TA	Con	364.17^{h}	383.15^{hi}	37.77^{abcdef}	6.77^{h}	21.79^{jklm}	208.43^{fgh}	10.45
	CR	277.38^{ijk}	295.23^{jk}	34.07^{abcdef}	6.83^{efg}	15.44^{no}	165.04^{ijklm}	9.36
	PO	236.48^{klm}	261.19^{klm}	33.83^{abcdef}	6.86^{cde}	12.93^{op}	139.93^{lmno}	9.24
96h of incubation								
AG	Con	554.89^{de}	567.97^{de}	40.49^{abcdef}	6.65^{jk}	40.30^{b}	282.35^{bc}	14.27
	CR	513.963^{c}	535.63^{e}	44.61^{abcdef}	6.68^{j}	36.64^{bc}	265.99^{cd}	13.78
	PO	384.04^{gh}	404.17^{ghi}	45.64^{abcde}	6.76^{hi}	26.62^{fghij}	210.50^{efg}	12.65
BD	Con	428.27^{f}	446.45^{f}	39.50^{abcdef}	6.73^{hi}	31.13^{def}	234.16^{def}	13.29
	CR	394.70^{fgh}	416.39^{fghi}	48.30^{abcd}	6.75^{hi}	25.85^{ghij}	214.55^{efg}	12.05
	PO	270.17^{ijkl}	296.21^{jk}	50.23^{abc}	6.84^{efg}	18.92^{mn}	165.04^{ijklm}	11.46
LP	Con	644.23^{a}	661.15^{a}	51.61^{ab}	6.54^{o}	49.21^{a}	347.01^{a}	14.18
	CR	623.77^{a}	646.55^{a}	44.66^{abcdef}	6.56^{no}	45.70^{a}	337.24^{a}	13.55
	PO	620.01^{ab}	640.92^{a}	41.15^{abcdef}	6.58^{mno}	45.19^{a}	336.45^{a}	13.43
TA	Con	412.23^{fg}	429.21^{fg}	48.14^{abcd}	6.73^{hi}	30.51^{efg}	234.84^{def}	12.99
	CR	285.21^{ij}	307.46^{ij}	49.73^{abc}	6.83^{efg}	19.92^{lmn}	173.21^{hijkl}	11.50
	PO	298.57^{i}	321.05^{j}	53.65^{ab}	6.81^{fg}	20.11^{lmn}	178.23^{ghijk}	11.28
SEM		25.8	25.8	2.0	0.02	1.6	12.8	

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM: Standard error of means; For: forage; AG: *A. gayanus*, BD: *B. decumbens*; LP: *L. perenne*; TA: *T. aestivum*; Con: control; CR: *C. rivulosus*; PO: *P. ostreatus*; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; NH_3-N: ammonia-nitrogen; CH_4: methane; tGP: total gas production.

The influence that PFF method produced on the main effects that fungus and incubation time had on the volatile fatty acids fraction (VFAs) of the 14 days pre-treated forages is presented in Table 60. The pre-treated forages varied significantly ($P < 0.05$) in the acetic, propionic, butyric, valeric, isovaleric and TVFA concentrations. Pre-treated *L. perenne* recorded the highest volatile fatty acids fractions, followed by *A. gayanus*, *B decumbens* and lastly by *T. aestivum*. The PFF method influenced the fungal effect on TVFAs of pre-treated forages, as they recorded reduced acetic, propionic, butyric, isovaleric, valeric and TVFA concentrations in comparison with the untreated forages. *P. ostreatus* treated forages recorded the lowest values for each of the volatile fatty acids molar fraction than *C. rivulosus* pre-treated forages. The volatile fatty acids increased with increase in the incubation time.

Table 60 The main effects of fungus and incubation time on the volatile fatty acids (mmol / L) content of 14 days pre-treated forages obtained from the PFF method

			Forage		
Parameters	***A. gayanus***	***B. decumbens***	***L. perenne***	***T. aestivum***	**SEM**
Acetic	81.49[b]	74.58[c]	116.40[a]	71.01[d]	1.72
Propionic	36.76[b]	33.06[c]	59.26[a]	30.36[d]	1.09
Butyric	10.98[b]	10.02[c]	22.09[a]	9.53[d]	0.47
Isovaleric	3.38[b]	3.34[b]	4.08[a]	3.05[b]	0.13
Valeric	1.83[b]	1.63[c]	2.29[a]	1.54[d]	0.04
TVFA	135.07[b]	123.16[c]	203.71[a]	116.13[d]	3.36
			Fungus		
Parameters	Control	*C. rivulosus*	*P. ostreatus*	SEM	
Acetic	92.01[a]	86.36[b]	79.25[c]	1.72	
Propionic	44.56[a]	39.86[b]	35.17[c]	1.09	
Butyric	14.58[a]	13.06[b]	11.83[c]	0.47	
Isovaleric	3.59[a]	3.45[a]	3.34[a]	0.13	
Valeric	1.93[a]	1.78[b]	1.75[b]	0.04	
TVFA	157.02[a]	144.91[b]	131.62[c]	3.36	
			Incubation time (hr)		
Parameters	24	48	96	SEM	
Acetic	76.51[c]	87.35[b]	93.76[a]	1.72	
Propionic	33.70[c]	40.78[b]	45.11[a]	1.09	
Butyric	11.00[c]	13.48[b]	14.99[a]	0.47	
Isovaleric	2.30[c]	2.96[b]	5.12[a]	0.13	
Valeric	1.44[c]	1.93[b]	2.10[a]	0.04	
TVFA	124.90[c]	147.60[b]	161.06[a]	3.36	

Means with different letters in the same row for each treatment are significantly ($P < 0.05$) different; SEM: Standard error of means.

Table 61 shows the influence that the PFF method produced on the interactive effects that fungus and incubation time had on the volatile fatty acids concentrations (mmol / L) of the14 days pre-treated and untreated forages. The volatile fatty acid molar concentrations of the untreated forages, as well as the pre-treated forages, increased ($P < 0.05$) with increase in the incubation time. The PFF method reduced the ability of the fungi to improve the TVFAs molar concentration of the pre-treated forages at each of the incubation times, in that it led to reduction in the TVFAs molar concentrations in comparison with those recorded in the un-treated forages. *P. ostreatus* pre-treated forages, at each incubation time, recorded more reduction in TVFA molar concentration than *C. rivulosus* treated ones, except in *T. aestivum* after 96h of incubation

Table 61 The interactive effects of fungus and incubation time on the volatile fatty acids (mmol / L) content of 14 days pre-treated forages obtained from the PFF method

		Volatile fatty acids (mmol / L)					
Forage	**Fungus**	**Acetic**	**Propionic**	**Butyric**	**Isovaleric**	**Valeric**	**TVFA's**
24h of incubation							
AG	Con	73.29^{j}	32.59^{lm}	10.06^{jklm}	2.47^{efgh}	1.47^{jkl}	119.88^{lm}
	CR	64.26^{lm}	24.81^{p}	8.13^{nopqr}	1.78^{h}	1.39^{klm}	101.24^{q}
	P0	63.67^{lm}	24.61^{p}	7.88^{opqr}	1.75^{h}	1.38^{klm}	100.06^{q}
BD	Con	70.02^{k}	29.91^{mno}	9.70^{klmno}	2.59^{defgh}	1.35^{klm}	113.57^{no}
	CR	69.68^{k}	29.46^{no}	9.40^{lmnop}	2.46^{efgh}	1.30^{klm}	113.26^{no}
	P0	58.65^{n}	21.81^{q}	6.96^{qr}	1.64^{h}	1.17^{m}	91.07^{r}
LP	Con	114.23^{c}	58.12^{cde}	19.71^{de}	$3.39^{abcdefgh}$	2.35^{c}	195.40^{d}
	CR	109.93^{d}	56.49^{de}	18.71^{e}	$3.16^{bcdefgh}$	1.56^{ghijk}	187.49^{e}
	P0	108.84^{d}	55.49^{e}	18.10^{e}	$3.13^{bcdefgh}$	1.54^{ghijk}	184.75^{ef}
TA	Con	69.35^{k}	29.31^{no}	9.58^{lmno}	1.96^{h}	1.36^{klm}	112.75^{o}
	CR	61.56^{mn}	23.18^{pq}	7.56^{pqr}	1.77^{h}	1.20^{lm}	96.16^{qr}
	P0	54.65^{o}	18.60^{r}	6.21^{r}	1.55^{h}	1.17^{m}	83.13^{s}
48h of incubation							
AG	Con	86.82^{gh}	42.61^{h}	12.15^{ghij}	3.22^{bcdefg}	1.96^{def}	148.88^{hi}
	CR	84.91^{ghi}	35.84^{k}	11.56^{hijk}	$3.19^{bcdefgh}$	1.94^{def}	138.46^{k}
	P0	72.17^{jk}	30.24^{lmno}	9.86^{klmn}	$3.07^{bcdefgh}$	1.74^{fghij}	118.04^{mno}
BD	Con	83.64^{hi}	40.65^{hij}	11.05^{ijkl}	$2.97^{bcdefgh}$	1.79^{fghi}	141.06^{jk}
	CR	81.97^{i}	38.79^{ij}	10.58^{ijkl}	2.91^{cdefgh}	1.77^{fghij}	137.04^{k}
	P0	62.59^{m}	23.91^{pq}	7.59^{pqr}	2.69^{defgh}	1.69^{fghij}	99.36^{q}
LP	Con	122.10^{a}	62.87^{ab}	25.65^{a}	$3.48^{abcdefgh}$	2.69^{ab}	217.95^{a}
	CR	116.42^{bc}	58.41^{cd}	21.34^{cd}	$3.35^{abcdefgh}$	2.35^{c}	203.04^{c}
	P0	117.18^{bc}	58.59^{cd}	22.88^{bc}	$3.41^{abcdefgh}$	2.40^{bc}	205.63^{bc}
TA	Con	82.32^{i}	38.91^{ij}	10.58^{ijkl}	2.62^{defgh}	1.77^{fghi}	137.39^{k}
	CR	72.02^{jk}	30.50^{lmno}	9.91^{klmn}	2.36^{fgh}	1.54^{hijk}	117.65^{mno}
	P0	66.03^{l}	28.01^{o}	8.59^{mnopq}	2.25^{gh}	1.50^{ijk}	106.58^{p}
96h of incubation							
AG	Con	105.27^{e}	52.43^{f}	15.08^{f}	$4.80^{abcdefg}$	2.17^{cde}	179.75^{f}
	CR	100.08^{f}	49.31^{g}	13.83^{fg}	5.04^{abcde}	2.21^{cd}	170.46^{g}
	P0	82.97i	38.39^{jk}	10.2^{3jklm}	5.06^{abcd}	2.22^{cd}	138.87^{k}
BD	Con	87.75^{g}	42.97^{h}	13.08^{gh}	$4.79^{abcdefg}$	1.84^{fg}	150.43^{h}
	CR	85.05^{ghi}	40.51^{hij}	12.05^{ghij}	4.98^{abcde}	1.89^{ef}	144.48^{ij}
	P0	71.90^{jk}	29.55^{no}	9.81^{klmno}	5.01^{abcde}	1.90^{ef}	118.17^{mn}
LP	Con	122.39^{a}	63.03^{a}	26.07^{a}	5.83^{a}	2.75^{a}	220.06^{a}
	CR	118.31^{b}	60.23^{bc}	23.38^{b}	5.50^{ab}	2.46^{abc}	209.88^{b}
	P0	118.18^{b}	60.13^{c}	22.97^{bc}	5.45^{abc}	2.42^{bc}	209.15^{b}
TA	Con	86.92^{g}	41.31^{hi}	12.25^{ghi}	4.98^{abcde}	1.71^{fghij}	147.17^{hi}
	CR	72.09^{jk}	30.75^{lmn}	10.23^{jklm}	4.90^{abcdef}	1.81^{fgh}	119.77^{lm}
	P0	74.15^{j}	32.66^{l}	10.86^{ijkl}	5.09^{abcd}	1.83^{fgh}	124.59^{l}
SEM		3.44	2.19	0.95	0.25	0.07	6.71

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM: Standard error of means; AG: *A. gayanus*, BD: *B. decumbens*; LP: *L. perenne*; TA: *T. aestivum*; Con: control; CR: *C. rivulosus*; PO: *P. ostreatus*; TVFAs: total volatile fatty acids.

5.3.4 *The degradability, fermentation profiles and total gas production of 28 days pre-treated forages obtained from the pump filtered method (PFF)*

The influence that PFF method produced on the main effects that fungus and incubation time had on the degradability and fermentation parameters of the twenty-eight days pre-treated forages is presented in Table 62. The forages recorded significant ($P < 0.05$) differences in their IVDMD and IVOMD, NH_3-N (mg/L), CH_4 (L/Kg OM), tGP (L / kg OM) and pH. The highest digestibility was recorded in pre-treated *L. perenne,* followed by *A. gayanus*, and lastly by *B. decumbens* and *T. aestivum*.

The PFF method influenced the fungal effect on the fermentation and digestibility parameters of the pre-treated forages, in that it led to a reduction in IVDMD and IVOMD, slight increase in NH_3-N content (mg / L), increase in pH, decrease in CH_4 and tGP respectively when compared to untreated forages. *P. ostreatus* pre-treated forages recording more reduction and increase in these components than *C. rivulosus* pre-treated forages. The influence that PFF method had on the incubation time was expected, as the parameters measured increased with increase in incubation time.

Table 62 The main effects of fungus, and incubation time on the degradability and fermentation parameters of 28 days pre-treated forages obtained from the PFF method.

	Forages				
Parameters	***A. gayanus***	***B. decumbens***	***L. perenne***	***T. aestivum***	**SEM**
IVDMD (g/kg DM)	355.83^{b}	331.60^{c}	570.19^{a}	333.83^{c}	11.24
IVOMD (g/kg DM)	374.01^{b}	354.97^{c}	596.37^{a}	358.37^{c}	11.51
NH_3-N (mg/L)	54.19^{a}	49.81^{b}	54.16^{a}	46.99^{b}	0.97
pH	6.77^{b}	6.79^{a}	6.62^{c}	6.79^{a}	0.01
CH_4 (L/kg OM)	23.23^{b}	21.32^{c}	34.78^{a}	21.41^{bc}	0.78
tGP (L/kg OM)	202.38^{b}	190.20^{c}	308.35^{a}	191.87^{c}	5.50
%CH_4/tGP	11.48	11.21	11.28	11.16	
	Fungi				
Parameters	**FC**	**CR**	**PO**	**SEM**	
IVDMD (g/kg DM)	440.26^{a}	397.42^{b}	355.90^{c}	11.24	
IVOMD (g/kg DM)	460.91^{a}	419.80^{b}	382.08^{c}	11.51	
NH_3-N (mg/L)	50.75^{b}	51.64^{a}	51.48^{a}	0.97	
pH	6.71^{c}	6.75^{b}	6.77^{a}	0.01	
CH_4 (L/kg DOM)	28.38^{a}	25.06^{b}	22.12^{c}	0.78	
tGP (L/Kg DOM)	243.41^{a}	224.63^{b}	201.56^{c}	5.50	
%CH_4/tGP	11.66	11.16	10.97		
	Incubation time (hrs)				
Parameters	24	48	96	SEM	
IVDMD (g/kg DM)	301.83^{c}	414.18^{b}	477.58^{a}	11.24	
IVOMD (g/kg DM)	320.62^{c}	437.38^{b}	504.80^{a}	11.51	
NH_3-N (mg/L)	43.32^{c}	47.98^{b}	62.57^{a}	0.97	
pH	6.82^{a}	6.73^{b}	6.68^{c}	0.01	
CH_4 (L/kg DOM)	14.59^{c}	25.03^{b}	35.94^{a}	0.78	
tGP (L/kg DOM)	178.24^{c}	231.33^{b}	260.03^{a}	5.50	
%CH_4/tGP	8.19	10.82	13.82		

Means with different letters in the same row for each treatment are significantly ($P < 0.05$) different; SEM, standard error of mean. SEM: Standard error of means; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; NH_3-N: ammonia-nitrogen; CH_4: methane; tGP: total gas production; CR: *C. rivulosus*; PO: *P. ostreatus*

Table 63 shows the influence that PFF method produced on the interactive effects that fungus and incubation time had on the nutrient degradability (IVDMD and IVOMD) and fermentation profiles (NH_3-N, CH_4, and pH) of the 28 days pre-treated forages. The influence of the PFF method on the interaction produced a significant effect ($P < 0.05$) on the IVDMD and IVOMD, NH_3-N (mg / L), pH, CH_4 (L/kg DM and OM), and tGP (L /kg OM) of the pre-treated and untreated forages. The IVDMD, IVOMD, NH_3-N, CH_4 production, tGP of the treated and untreated forages increased, while the pH decreased with increase in incubation time. The PFF method had a negative influence on the ability of the fungi to improve the digestibility and fermentation of the pre-treated forages as it reduced the IVDMD, IVOMD and NH_3-N content, increased the pH, reduced the CH_4 production and tGP at each incubation time compared to the untreated forages. The only exception was the higher NH_3-N content recorded in almost all of the pre-treated forages at 96h of incubation in comparison with the untreated forages. Most of the *P. ostreatus* pre-treated forages recorded lower IVDMD and IVOMD, lower CH_4 and tGP, lower NH_3-N content and higher pH than *C. rivulosus* pre-treated forages.

Table 63 The interactive effects of fungus and incubation time on the degradability and fermentation parameters of 28 days pre-treated forages obtained from the PFF method

For	Fun	IVDMD g/kg DM	IVOMD g/kg OM	NH_3-N (mg/L)	pH	CH_4 L/kg DOM)	tGP L/kg DOM)	$\%CH_4$/ TGP
24h of incubation								
AG	Con	296.60^{hijkl}	308.12^{ijkl}	49.55^{defg}	6.82^{defgh}	14.70^{ijklm}	175.98^{ijkl}	8.35
	CR	215.47^{op}	229.30^{mn}	47.34^{defg}	6.88^{b}	10.00^{lm}	140.55^{mn}	7.11
	PO	222.63^{nop}	241.26^{lmn}	46.73^{defg}	6.87^{bc}	10.60^{lm}	148.30^{m}	7.15
BD	Con	266.85^{klmno}	286.72^{jklm}	43.90^{efg}	6.83^{bcdef}	12.80^{jklm}	171.33^{ijklm}	7.47
	CR	225.30^{mnop}	252.94^{lmn}	37.16^{g}	6.87^{bc}	10.72^{lm}	150.14^{lm}	7.14
	PO	216.11^{op}	236.93^{mn}	35.88^{g}	6.88^{b}	10.08^{lm}	141.60^{mn}	7.12
LP	Con	595.99^{a}	618.73^{a}	46.58^{defg}	6.61^{mn}	31.64^{cde}	324.05^{ab}	9.76
	CR	466.13^{b}	486.30^{b}	43.99^{efg}	6.70^{l}	22.00^{ghi}	256.67^{cd}	8.57
	PO	455.30^{b}	484.99^{b}	43.00^{efg}	6.71^{l}	21.07^{ghij}	244.93^{de}	8.60
TA	Con	237.66^{lmno}	253.48^{klm}	42.85^{efg}	6.85^{bcd}	11.30^{klm}	156.40^{klm}	7.23
	CR	256.93^{klmno}	274.59^{jklm}	44.83^{defg}	6.84^{bcde}	12.40^{klm}	165.10^{jklm}	7.51
	PO	167.57^{p}	184.03^{n}	37.98^{fg}	6.945^{a}	7.72^{m}	103.36^{n}	7.47
48h of incubation								
AG	Con	419.71^{bcde}	436.15^{bcde}	52.47^{bcdefg}	6.74^{jkl}	27.47^{defg}	236.41^{def}	11.62
	CR	353.91^{efghi}	370.74^{efghi}	50.39^{cdefg}	6.78^{ghijk}	22.36^{fghi}	197.76^{fghij}	11.31
	PO	319.85^{ghijk}	339.27^{ghij}	48.77^{defg}	6.80^{efghi}	19.30^{gijk}	187.54^{hijk}	10.29
BD	Con	412.73^{bcde}	435.35^{bcde}	48.67^{defg}	6.74^{jkl}	26.27^{efg}	231.68^{defg}	11.34
	CR	346.23^{fghij}	366.01^{ghi}	38.72^{fg}	6.78^{fghij}	21.10^{ghij}	194.90^{ghij}	10.83
	PO	283.13^{jklmn}	306.17^{ijkl}	44.75^{defg}	6.83^{cdefg}	16.95^{hijkl}	168.71^{ijkl}	10.05
LP	Con	612.83^{a}	634.55^{a}	54.39^{bcdefg}	6.59^{no}	36.25^{bc}	331.01^{a}	10.95
	CR	581.80^{a}	606.83^{a}	52.06^{bcdefg}	6.63^{mn}	32.89^{cde}	315.37^{ab}	10.43
	PO	587.00^{a}	624.47^{a}	52.04^{bcdefg}	6.62^{mn}	33.35^{cde}	317.86^{ab}	10.49
TA	Con	358.84^{defgh}	376.90^{defgh}	45.43^{defg}	6.77^{hijk}	22.35^{fghi}	201.04^{fghij}	11.12
	CR	405.07^{bcdef}	433.90^{bcdef}	47.23^{defg}	6.75^{ijkl}	25.25^{efgh}	225.18^{defgh}	11.21
	PO	289.04^{ijklm}	315.22^{hijk}	40.79^{efg}	6.82^{defgh}	16.85^{ijkl}	168.55^{ijkl}	10.00
96h of incubation								
AG	Con	565.68^{a}	586.99^{a}	59.06^{abcde}	6.64^{m}	43.87^{ab}	289.68^{bc}	15.14
	CR	435.91^{bc}	455.68^{bc}	62.94^{abcd}	6.72^{l}	33.50^{cde}	246.47^{d}	13.59
	PO	373.30^{cdefg}	398.62^{cdefg}	70.48^{ab}	6.74^{jkl}	27.32^{efg}	205.34^{efghi}	13.30
BD	Con	468.69^{b}	492.99^{b}	53.85^{bcdefg}	6.70^{l}	36.41^{bc}	246.65^{d}	14.76
	CR	430.32^{bc}	460.38^{bc}	68.83^{abc}	6.73^{kl}	31.33^{cde}	236.35^{def}	13.26
	PO	335.03^{ghij}	367.22^{fghi}	76.55^{a}	6.78^{ghijk}	26.20^{efgh}	186.45^{hijk}	14.05
LP	Con	626.90^{a}	649.61^{a}	56.21^{bcdef}	6.56^{o}	46.84^{a}	335.20^{a}	13.97
	CR	589.75^{a}	612.62^{a}	68.71^{abc}	6.62^{mn}	43.41^{ab}	320.66^{ab}	13.54
	PO	616.01^{a}	646.27^{a}	70.47^{ab}	6.58^{no}	45.57^{a}	334.88^{a}	13.61
TA	Con	421.27^{bcd}	448.27^{bc}	56.00^{bcdef}	6.73^{kl}	30.61^{cdef}	235.53^{defg}	13.00
	CR	462.24^{b}	498.36^{b}	57.49^{bcde}	6.71^{l}	35.77^{bcd}	258.86^{cd}	13.82
	PO	405.81^{bcdef}	440.54^{bcd}	50.30^{cdefg}	6.74^{jkl}	30.47^{cdef}	224.28^{defghij}	13.59
SEM		22.49	23.02	1.94	0.02	1.56	10.99	

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM: Standard error of means; For: forage; Fun: fungi; AG: *A. gayanus*, BD: *B. decumbens*; LP: *L. perenne*; TA: *T. aestivum*; Con: control; CR: *C. rivulosus*; PO: *P. ostreatus*; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; NH_3-N: ammonia-nitrogen; CH_4: methane; tGP: total gas production.

The influence that PFF method produced on the main effects that fungus and incubation time had on the VFAs of the 28 days pre-treated forages is presented in Table 64. The pre-treated forages varied significantly ($P < 0.05$) in the acetic, propionic, butyric, valeric, isovaleric and TVFA concentrations. Pre-treated *L. perenne* recorded the highest volatile fatty acid fractions, followed by *A. gayanus*, *B decumbens* and lastly by *T. aestivum*. The PFF method influenced the fungal effect on TVFAs of pre-treated forages, as they recorded reduced acetic, propionic, butyric, isovaleric, valeric and TVFA concentrations in comparison with the untreated forages. *P. ostreatus* treated forages recorded the lowest values for each of the volatile fatty acids molar fraction than *C. rivulosus* pre-treated forages. The volatile fatty acids increased with increase in the incubation time.

Table 64 The main effects of fungus and incubation time on the volatile fatty acid contents of 28 days pre-treated forages obtained from the PFF method.

			Forage		
Parameters	***A. gayanus***	***B. decumbens***	***L. perenne***	***T. aestivum***	**SEM**
Acetic	80.43^{b}	78.42^{c}	110.86^{a}	77.61^{c}	1.62
Propionic	36.28^{b}	34.96^{c}	53.06^{a}	35.01^{c}	0.89
Butyric	10.37^{b}	10.16^{bc}	19.76^{a}	10.07^{c}	0.38
Isovaleric	2.87^{b}	2.90^{b}	3.22^{a}	2.90^{b}	0.04
Valeric	1.57^{bc}	1.62^{b}	1.80^{a}	1.54^{c}	0.02
TVFA	131.60^{b}	128.09^{c}	188.68^{a}	127.18^{c}	2.89
			Fungus		
Parameters	Control	*C. rivulosus*	*P. ostreatus*	SEM	
Acetic	92.70^{a}	86.90^{b}	80.88^{c}	1.62	
Propionic	42.99^{a}	39.69^{b}	36.80^{c}	0.89	
Butyric	13.42^{a}	12.46^{b}	11.89^{c}	0.38	
Isovaleric	3.02^{a}	2.97^{ab}	2.93^{b}	0.04	
Valeric	1.67^{a}	1.64^{b}	1.59^{b}	0.02	
TVFA	153.79^{a}	143.74^{b}	134.14^{c}	2.89	
		Incubation times (hr)			
Parameters	24	48	96	SEM	
Acetic	71.67^{c}	89.29^{b}	99.53^{a}	1.62	
Propionic	47.10^{a}	40.56^{b}	31.81^{c}	0.89	
Butyric	10.72^{c}	12.77^{b}	14.29^{a}	0.38	
Isovaleric	2.44^{c}	3.16^{b}	3.32^{a}	0.04	
Valeric	1.36^{c}	1.68^{b}	1.86^{a}	0.02	
TVFA	118.04^{c}	147.52^{b}	166.10^{a}	2.89	

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM, standard error of mean. SEM: Standard error of means.

Table 65 shows the influence that PFF method produced on the interactive effects that fungus and incubation time had on the VFAs concentrations (mmol / L) of the 28 days pre-treated and untreated forages. The volatile fatty acid molar concentrations of the untreated forages, as well as the pre-treated forages, increased ($P < 0.05$) with increase in the incubation time. The PFF method reduced the ability of the fungi to improve the TVFAs molar concentration of the pre-treated forages at each of the incubation times, in that it led to reduction in the TVFAs molar concentrations in comparison with those recorded in the un-treated forages. *P. ostreatus* pre-treated forages, at each incubation time, recorded more reduction in TVFA molar concentration than *C. rivulosus* treated ones, except in *L. perenne*.

Table 65 The interactive effect of fungus and incubation time on the volatile fatty acids content of 28 days pre-treated forages obtained from the PFF method

		Volatile fatty acids (mmol / L)					
Forage	**Fungi**	**Acetic**	**Propionic**	**Butyric**	**Isovaleric**	**Valeric**	**TVFAs**
24h of incubation							
AG	Con	72.30^{op}	31.58^{m}	9.18^{op}	2.24^{jk}	1.41^{jklmn}	116.71^{mn}
	CR	57.78^{s}	24.51^{p}	8.40^{pq}	2.19^{jk}	1.30^{lmn}	94.17^{r}
	P0	59.74^{s}	25.90^{op}	8.87^{p}	2.16^{k}	1.30^{lmn}	97.96^{r}
BD	Con	68.65^{pqr}	29.04^{n}	9.02^{p}	2.43^{hijk}	1.44^{ijklmn}	110.58^{op}
	CR	60.14^{s}	26.17^{sp}	8.95^{p}	2.24^{jk}	1.32^{lmn}	98.83^{r}
	P0	59.23^{s}	25.65^{op}	8.85^{p}	2.18^{jk}	1.27^{mn}	97.18^{r}
LP	Con	109.59^{de}	52.76^{b}	18.91^{d}	3.11^{cde}	$1.54^{fghijklm}$	185.91^{d}
	CR	97.33^{f}	44.97^{d}	16.13^{e}	2.70^{fgh}	$1.50^{ghijklmn}$	162.63^{f}
	P0	95.67^{fg}	43.51^{def}	15.09^{f}	2.62^{ghi}	1.41^{jklmn}	158.30^{fg}
TA	Con	64.97^{r}	27.74^{no}	8.66^{pq}	2.47^{hijk}	1.32^{lmn}	105.15^{q}
	CR	66.60^{qr}	29.03^{n}	8.88^{p}	2.53^{hij}	1.35^{klmn}	108.54^{pq}
	P0	48.00^{t}	20.88^{q}	7.70^{q}	2.35^{ijk}	1.22^{n}	80.52^{s}
48h of incubation							
AG	Con	90.11^{hij}	41.35^{fg}	11.60^{hijk}	3.23^{bcde}	$1.67^{defghij}$	147.96^{ij}
	CR	82.19^{mn}	36.08^{jk}	10.50^{lmn}	3.18^{bcde}	$1.64^{defghij}$	134.69^{l}
	P0	73.76^{o}	33.61^{lm}	9.20^{op}	2.97^{ef}	$1.54^{fghijklm}$	121.29^{m}
BD	Con	88.30^{ijk}	40.34^{gh}	11.04^{jklmn}	3.19^{bcde}	1.74^{cdefgh}	144.61^{jk}
	CR	82.16^{n}	35.11^{kl}	10.32^{mn}	2.96^{efg}	$1.57^{efghijkl}$	132.36^{l}
	P0	70.18^{opq}	31.50^{m}	9.15^{p}	3.12^{cde}	$1.63^{defghijk}$	115.58^{no}
LP	Con	117.61^{ab}	57.26^{a}	21.42^{b}	3.38^{abcd}	1.90^{abcd}	201.58^{b}
	CR	111.33^{cd}	53.60^{b}	19.53^{d}	3.31^{bcde}	1.86^{bcde}	189.62^{cd}
	P0	114.06^{bc}	54.57^{b}	19.89^{cd}	3.24^{bcde}	1.71^{defghi}	193.47^{c}
TA	Con	83.75^{lmn}	32.36^{m}	10.67^{klmn}	3.11^{cde}	$1.68^{defghij}$	131.57^{l}
	CR	86.01^{klm}	38.78^{hi}	10.68^{klmn}	3.14^{cde}	1.71^{defghi}	140.32^{k}
	P0	71.37^{op}	32.19^{m}	9.17^{op}	3.06^{de}	$1.46^{hijklmn}$	117.24^{mn}
96h of incubation							
AG	Con	107.14^{e}	53.81^{b}	13.54^{g}	3.26^{bcde}	1.74^{cdefgh}	179.49^{e}
	CR	93.07^{gh}	42.22^{efg}	11.60^{hijk}	3.28^{bcde}	1.76^{cdefgj}	151.92^{hi}
	P0	87.15^{jkl}	37.43^{ij}	10.47^{lmn}	3.33^{abcd}	1.81^{cdef}	140.20^{k}
BD	Con	98.39^{f}	47.21^{c}	12.25^{h}	3.26^{bcde}	1.83^{cde}	162.93^{f}
	CR	95.77^{fg}	44.00^{de}	11.71^{hij}	3.35^{abcd}	1.85^{bcde}	156.68^{gh}
	P0	83.01^{mn}	35.63^{jkl}	10.18^{hij}	3.36^{abcd}	1.88^{abcd}	134.05^{l}
LP	Con	120.34^{a}	58.67^{a}	23.30^{a}	3.45^{abc}	2.01^{abc}	207.76^{a}
	CR	112.68^{cd}	53.95^{b}	20.60^{bc}	3.49^{ab}	2.12^{ab}	192.85^{c}
	P0	119.09^{a}	58.21^{a}	22.92^{a}	3.66^{a}	2.14^{a}	206.02^{ab}
TA	Con	91.26^{hi}	43.73^{de}	11.470^{hijkl}	3.13^{cde}	1.72^{defgh}	151.29^{i}
	CR	97.19^{f}	47.85^{c}	12.20^{hi}	3.25^{bcde}	1.74^{cdefgh}	162.23^{f}
	P0	89.32^{ijk}	42.54^{ef}	11.21^{ijklm}	3.07^{de}	$1.66^{defghij}$	147.80^{ij}
SEM		3.23	1.78	0.76	0.07	0.04	5.77

Means with different letters in the same column are significantly ($P < 0.05$) different; SEM: Standard error of means; AG: *A. gayanus*, BD: *B. decumbens*; LP: *L. perenne*; TA: *T. aestivum*; Con: control; CR: *C. rivulosus*; PO: *P. ostreatus*; IVDMD: *in vitro* dry matter degradability; IVOMD: *in vitro* organic matter degradability; tGP: total gas production

5.4 Discussion

This study investigated the influence of the loss of solubles (i.e. NDS and WSC) from fungal treated forages on the ability of fungi to improve nutrient composition and *in vitro* degradability of pre-treated forages. In the practical sense, the loss of solubles happens when farmers uses sacks/bags/container with pores for the pre-treatment and in addition to this some puts heavy stones on these containers which leads to further pressure on the pre-treated substrates. The means of soluble losses were then mimicked in the laboratory using two filtration methods (i.e. PFF and FFF) However, to facilitate the release of solubles in the pre-treated forages so as to achieve the aim of this study, two treatment factors, i.e. substrate-liquid ratio and incubation time were used. In this study, it can be deduced that loss of more solubles through the PFF method as opposed to the FFF method did not improve the chemical composition, *in vitro* nutrient degradability and *in vitro* fermentation parameters of the fungal-pre-treated forages. It appeared that, the removal of solubles caused increases in the concentration of insoluble components in the pre-treated forages, that had a negative influence on their degradability and fermentation parameters. The degradability of the pre-treated forages obtained from the FFF method was not determined but, based on its more soluble components, the FFF method can be predicted to improve the digestibility of untreated and pre-treated forages more than the PFF method . It can also be deduced that the substrate-liquid ratio of 1:5 supported increased activity and growth of *C. rivulosus* on all the forages, and *P. ostreatus* on some forages except *A. gayanus* and *B. decumbens*, with the release of solubles from which more losses were recorded when the filtration methods were applied. It can be deduced that short inoculation time supported both fungal growth and activity on *L. perenne* and *T. aestivum* but not on *A. gayanus* and *B. decumbens* for increased release of solubles.

5.4.1 *Fungal growth performance and laccase activity influence on nutrient loss*

The investigation of the fungal growth and laccase activity using 2 treatment conditions, i.e. substrate-liquid ratio and incubation time, was carried out to determine the best substrate-liquid ratio and incubation time that supports increased fungal activity and growth from which more solubles are expected to be released. This is because the solubles (i.e. NDS and WSC) were products of increased fungal growth and activity in anticipation for degradation of structural components. The increased laccase activity and growth of *C. rivulosus* on all the forages, as well as *P. ostreatus* on some forages except *A. gayanus* and *B. decumbens,* when using a substrate-liquid ratio of 1:5 was an indication that this condition was optimal for their growth and activity. Optimal moisture content has been reported to positively influence fungal growth and laccase production by preventing substrate porosity or agglomeration, thus allowing

oxygen transfer that is required for the exhibition of fungal ligninolytic activities (Molin *et al.*, 1992; Gowthaman *et al.*, 2001; Krishna, 2005; Bhargav *et al.*, 2008; Patel *et al.*, 2009). In addition, during the colonization of substrate by fungi, they first of all metabolise digestible carbohydrates such as hemicellulose into soluble sugars, as they require these easily accessible metabolisable compounds to initiate their growth and enzymatic function (Krishna, 2005; Mishra and Kumar, 2007; Zheng and Rehmann, 2014; Daniel, 2016) in preparation for degradation (Karunanandaa *et al.*, 1995). Therefore at this stage of growth, it is expected that the substrates contain more solubles. To examine the loss by drainage of these solutes, the filtration methods were applied, to more effectively investigate the impact that this will have on the degradability of pre-treated forages. This makes the use of pre-treated substrates in this liquid ratio a good choice for the degradability experiment.

The shorter incubation time that supported laccase activity and growth on *L. perenne* and *T. aestivum,* as opposed to the longer incubation time that supported fungal growth and activity on *B. decumbens* and *A. gayanus,* is an indication that forages used for fungal treatment played a significant role in the liberation of solubles (Lonsane *et al.*, 1985; Gowthaman *et al.*, 2001; Krishna, 2005; Bhargav *et al.*, 2008). Several factors such as their nutrient composition (Krishna, 2005; Zheng and Rehmann, 2014), degree of crystallinity of structural components especially cellulose (Karunanandaa *et al.*, 1995; Mahesh and Mohini, 2013), differences in the cell wall composition of C_3 and C_4 plants (Akin, 1989; Lengowski *et al.*, 2016), forage particle size (van Kuijk *et al.*, 2016b) etc. did have an influence on the ability of the fungi to get the required nutrients needed to initiate their growth and activity (Mishra and Kumar, 2007; Daniel, 2016). Furthermore, fungi also vary in the incubation time at which they liberate solubles from substrates in preparation for lignocellulose degradation (Sharma and Arora, 2015). Arora and Sharma (2009a) investigated different fungal species over various times for the liberation of maximum water solubles and found the following observations: 20 days (*C. subvermispora*) with no significant increase at an extended incubation time; 30 days (*Phlebia fascicularia* and *Phlebia brevispora*); and 10 days (*Phlebia radiata*). Therefore the application of filtration methods both at the shorter and longer incubation time for investigating its influence on the nutrient composition and degradability is justified, as the forages tested varied in their responses to fungal growth and activity at different incubation times. Also with the particle size (2mm) used in this study, early fungal colonization is expected, as smaller particle size facilitates fungal colonization by exposing more sites for them to grow on and carry out their enzymatic activity within the shortest period of time (Krishna, 2005; van Kuijk *et al.*, 2016b), therefore justifying the shorter inoculation times (i.e. 14 and 28 days) used. This is also supported by

previous findings were shorter inoculation time that ranges between 14-35 days were used for pre-treatment of small particle size (1mm -3mm) substrates (Hakala *et al.*, 2005; Hildén *et al.*, 2007; Akinfemi, 2010a). Although the use of smaller particle size substrate can interfere with oxygen penetration into the substrate which can negatively affect fungal growth and activity, however this is also dependent on the substrate characteristics and the capacity of the system.

The increased laccase activity and growth exhibited by *P. ostreatus* in comparison with *C. rivulosus* when inoculated on most forages except *L. perenne* is an indication that different species of fungi vary in their activity, depending on the available substrates (Krishna, 2005; Patel *et al.*, 2009). *P. ostreatus* is widely known for its potential in producing a significant amount of laccase enzymes on substrates, as reported by previous researchers (Stajić *et al.*, 2006a; Patel *et al.*, 2009). *C. rivulosus* is also known to produce laccase enzyme, but in most cases its production is dependent on the substrate nutrient composition. Hakala *et al.* (2005) discovered no laccase production in *C. rivulosus* when grown on spruce wood chips, but produced a more substantial amount of manganese peroxidase. However, when the crude protein extract was concentrated to monitor the production of laccase isoforms, the laccase enzyme was then visible. This might be the reason why *C. rivulosus* exhibited more growth on *L. perenne* as this contained the highest nitrogen content. Therefore, *P. ostreatus* pre-treated forages are expected to produce more solubles than *C. rivulosus* treated ones, except *L. perenne*, and if the filtration method is applied, a greater soluble loss might be recorded.

5.4.2 *Influence of filtration methods on the chemical composition of pre-treated forages*

The greater soluble losses in the pre-treated forages using the PFF method as opposed to the FFF method results in a greater negative impact on the fungal ability to improve the chemical composition of 14 and 28 days pre-treated forages in 2 different liquid ratios. This supports the findings of Arora and Sharma (2009b) and Zuo *et al.* (2018), who identified that solubles (i.e. NDS and WSC) were needed to increase the nutritive composition of pre-treated forages and their loss is detrimental to the improvement of the chemical composition of pre-treated forages.

The increased CP content of pre-treated forages obtained from both filtration methods was not expected. The expectation was that the CP content of the pre-treated forages from PFF would be low and that of the FFF would be higher, as CP was regarded as part of the solubles; however, this was not the case. This shows that the absolute protein content of the forages might not have changed, but the added fungal biomass (i.e. mycelium/chitin) which was found in both pre-treated forages obtained from both methods might have contributed to the increased CP content recorded. This supports the findings that increased fungal biomass on the substrate is associated

with the increased CP content (Chen *et al.*, 1995; Fazaeli, 2007; Di Mario *et al.*, 2008; Niu *et al.*, 2018; Zuo *et al.*, 2018). The result was in agreement with other research findings where fungal treatments led to increased CP content of substrates (Díaz and C. Sánchez, 2002; Fazaeli, 2007; Akinfemi, 2010b; Sharma and Arora, 2010a; Shrivastava *et al.*, 2012; Tuyen *et al.*, 2012).

The reduced OM content in pre-treated forages obtained from both filtration methods was expected, as fungi utilised OM content for their growth and activity, thus increasing ash and CP contents (Chang *et al.*, 2012; Zuo *et al.*, 2018). However, the expectation was that the pre-treated forages obtained from the PFF method would show more OM loss, as these recorded more soluble losses than the pre-treated forages obtained from the FFF method, but this was not the case. This can be linked to the fact that the FFF method still retained the solubles as well as the ash content but on the other hand the PFF method removed most of the solubles and ash content, thus increasing the percentage of OM content in the leftover substrates.

The reduction in structural components (i.e. fibre and predicted lignin contents) of the pre-treated forages obtained from the FFF method as against the PFF method indicates that minimal solubles are lost with this method and the obtained results were in agreement with previous findings where reduction in fibre composition was recorded in fungal pre-treated forages (Díaz and C. Sánchez, 2002; Rodrigues *et al.*, 2008; Akinfemi, 2010b; Abdel-Azim *et al.*, 2011; Tuyen *et al.*, 2013; Niu *et al.*, 2018; Zuo *et al.*, 2018). This further ascertained that loss of solubles through any form of pressure is detrimental to the improvement of pre-treated forages and this must be avoided when handling pre-treated forages.

The reduction in the secondary metabolite contents of the pre-treated forages obtained from both PFF and FFF methods is an indication that loss of solubles using any form of filtration method has little effect on metabolite contents of treated forages, as the FFF pre-treated forages were similar to the PFF pre-treated forages. This is an indication that the fungi are detoxifying/mineralising and removing reactive polycyclic aromatic complexes and phenolic compounds through their laccase enzymatic polymerization function and using them for their growth on different substrates (Annachhatre and Gheewala, 1996; Pérez *et al.*, 2002; Niladevi and Prema, 2008). In addition, the expectation was that the pre-treated forages obtained from the FFF method would increase the metabolite contents, as lignin degradation took place with minimal soluble loss compared to the PFF method. The degradation of lignin phenolic covalent bonds was expected to be accompanied with the release of more phenolic compounds and total antioxidant contents, as identified in the findings of Lateef *et al.* (2008), Arora *et al.* (2011) and Pouteau *et al.* (2003), however, this was not the case. The difference might be attributed to the

lignin molecular weight/ group (Pan *et al.*, 2006), as lignin with less aliphatic, more phenolic hydroxyl groups, wide poly disparity and high molecular weight, does lower the ability of the treated forages to be improved in terms of total phenolic and total antioxidant contents (Pan *et al.*, 2006).

The more pronounced inability of the used fungi to improve the nutrient composition of most of the pre-treated forages obtained from the PFF method, as opposed to those derived from the FFF method, when a shorter inoculation time and a substrate-liquid ratio of 1:5 were used can be attributed to two major reasons. Firstly, it might be that the fungal activity and growth was increased under these conditions, which eventually led to the release of more solubles that were then lost through the filtration method. Secondly, it might be that an extensive lignocellulose degradation has not taken place under these conditions for a considerable amount of improvement to be recorded, coupled with the soluble loss caused through the PFF method. This supports the existing findings that inoculation time plays a significant role in lignocellulose degradation, as fungi vary in the time required for the release of solubles which are utilised by fungi for growth in preparation for lignocellulose degradation (Tuomela *et al.*, 2002; Arora and Sharma, 2009a; Abdel-Azim *et al.*, 2011). Also, it supports the findings that obtaining the optimum initial moisture level has a pronounced effect on adequate utilisation of substrate and the ligninolytic enzymatic activity of the fungi (Patel *et al.*, 2009) required for efficient lignocellulose degradation. The increased OM, hemicellulose and carbon contents of most of the pre-treated forages obtained from the PFF and FFF methods at shorter inoculation time were a confirmation that an extensive lignocellulose degradation had not taken place, as fungi consumed these nutrients for their growth and activity (Rodrigues *et al.*, 2008; Arora and Sharma, 2009a; nee'Nigam *et al.*, 2009).

P. ostreatus might have recorded higher soluble loss than *C. rivulosus* at both inoculation times, as it exhibited higher lignocellulose degradation in most of the pre-treated forages. This is a reflection that fungi vary in the rate at which lignocellulosic degradation is achieved through enzymatic functions, and this is evident in the quantity of soluble contents liberated (Arora and Sharma, 2009a; Giles *et al.*, 2015; Maza *et al.*, 2015). Thus, a greater soluble loss is expected in *P. ostreatus* treated forages at both inoculation times, but more pronounced with a longer inoculation time, than *C. rivulosus* treated ones. The increased soluble loss is expected to be complemented with an increase in insoluble fractions, i.e. fibre and lignin, however this was not the case in the 28th day pre-treated forages. The possibility might be that, at this extended pre-treatment time, a less amount of soluble was available. Therefore, the pronounced effect on structural components could not be recorded through the filtration method, although some solubles were still expected to be removed due to the slight pressure by the pump. The other

possibility might be that, as the inoculation time increased, the pre-treated forages became porous, therefore reducing the drastic effect that the filtration method might have exerted on the fibre composition. Biological pre-treatment of a substrate has been found to increase the porous nature of pre-treated substrate in readiness for further enzymatic degradation of lignocellulose (Taniguchi *et al.*, 2005; Yu *et al.*, 2009a).

The increased nutrient losses (i.e. fibre, lignin and metabolites) and nutrient gain (CP and ash) in pre-treated forages obtained from the FFF method rather than the PFF method using both inoculation times and substrate-liquid ratios were not expected. The expectation was that the PFF method would give more nutrient loss, since the method has supported more soluble loss. This might be that the leftover substrate no longer reflect the right composition of what might normally be expected if not pump filtered. The obtained results from the FFF method were in agreement with other research findings (Vane *et al.*, 2001; Tuyen *et al.*, 2012; Tuyen *et al.*, 2013). The CP and ash gain have been complemented with fibre loss, and this is in support of the findings of Akinfemi (2010b) and Tuyen *et al.* (2013). The DM losses recorded in this study were less than the DM losses range of 6 - 40% and 4.4 – 18.4% as reported by Agosin and Odier (1985) and Tuyen *et al.* (2013) when several organisms were used on different substrates. The differences might be attributed to fungal species, the substrate used, moisture content, length of fermentation period, temperature, environmental conditions and particle size etc.(Gowthaman *et al.*, 2001; Krishna, 2005; Patel *et al.*, 2009). Hemicellulose loss can be attributed to the fact that most fungi utilise hemicellulose for their initial mycelium growth and/or sporulation before they carry out their ligninolytic functions (Rodrigues *et al.*, 2008). Secondary metabolite loss can also be attributed to the fungi utilising this for their growth and activity (Pérez *et al.*, 2002; Niladevi and Prema, 2008). The obtained results were not in agreement with the findings of Assi and King (2007) and (Lateef *et al.*, 2008) were they recorded gain in the phenolic compounds (i.e. total antioxidant) content of some pre-treated agricultural residues and tomato pomace. This may be because the phenolic compounds constituents were not toxic to the fungi, thus inducing mediators that facilitated the fungal ligninolytic enzymatic potential for increased detoxification of these compounds (Karunanandaa *et al.*, 1995; Pérez *et al.*, 2002; Irshad *et al.*, 2011). The exhibition of more nutrient loss by *P. ostreatus* than *C. rivulosus* indicates that *P. ostreatus* degrading potential is high (Jung *et al.*, 1992; Arora and Sharma, 2009a; Patel *et al.*, 2009) or that it might have to use the nutrients for its mycelium growth (nee'Nigam *et al.*, 2009). The filtration method has been identified to have influenced the ability the fungi had to improve the chemical composition of the pre-treated forages under the growth conditions

selected; the PFF method influenced it negatively while the FFF method influenced it positively.

5.4.3 *Degradability, fermentation profiles and total gas production of pre-treated forages obtained from the pump filtered (PFF) method.*

This study has been able to ascertain the involvement of solubles in degradability/digestibility when pre-treated samples in a substrate-liquid ratio of 1:5 from both 14 day and 28 days of fungal pre-treatment were used. The PFF method reduced the ability of the fungi to improve nutrient degradability/ digestibility and the fermentation parameters. Although the pre-treated forages obtained through the FFF method were not investigated, it would have been capable of increasing the ability of the fungi towards improving nutrient degradability/ digestibility as it was close to the usual situation of handling pre-treated forages. The PFF method caused a higher loss of solubles and an increase in the insoluble fractions of the pre-treated forages, which led to a reduction in their degradability and fermentation parameters when investigated.

The reduction in nutrient degradability (i.e. IVOMD and IVDMD) can be attributed to the chemical composition of the pre-treated forages, as they recorded higher CP and ash contents that was complemented with higher fibre (i.e. NDF and ADF) and lignin contents than the untreated forages. Fibre, especially ADF, has been reported to be negatively correlated to digestibility as it contains indigestible cell wall contents (i.e. lignin and cellulose) that are usually resistant to rumen microbial degradation (Van Saun, 2006; Akinfemi, 2010b; Mahesh and Mohini, 2013). Furthermore, the increased ash content might also have reduced nutrient degradability, as ash has been found to be negatively correlated to degradability (Sharma and Arora, 2010a). The increased CP content of the pre-treated forages didn't influence the nutrient degradability positively, indicating that the absolute CP amount did not change but the increase was from the addition of fungal biomass/chitin. Fungal chitin polymers are usually resistant to degradability (Husby *et al.*, 1981; Einbu and Vårum, 2008; Nayan *et al.*, 2018) and might have caused further reduction in their degradation. Also, it was reported by a researcher that the principle involved in upgrading lignocellulosic substrates into protein-rich substrates was found to be antagonistic to the increased degradability of the substrate (Villas-Bôas *et al.*, 2002). The other factors that might have reduced the degradability include fungal species (Akinfemi, 2010b), excessive degradation of lignocellulose by fungi (Karunanandaa *et al.*, 1995) etc. However, these influences would have been more pronounced if the conditions for the handling of the pre-treated forages were the ideal conditions, like in the case of pre-treated forages

obtained from the FFF method. However, due to availability of time, the FFF samples were not investigated for *in vitro* studies.

The increased nutrient degradability of some of the 14 days pre-treated forages (i.e. *A. gayanus* and *L. perenne*) in comparison to those obtained from 28 days pre-treatment might be linked to their hemicellulose content, the degree of crystallinity of the leftover substrate and forages. The hemicellulose content of most of the pre-treated forages obtained after the shorter inoculation time was higher than those obtained after the longer inoculation time, and hemicellulose is found to be easily hydrolysable by rumen microbes (Pérez *et al.*, 2002). The other point is that there is the possibility that the fungal extensive lignocellulose degradation has removed part of the structural carbohydrates, therefore, leaving behind the most recalcitrant and crystalline structural carbohydrates that usually resist rumen microbial degradation (Karunanandaa *et al.*, 1995) in the 28 days pre-treated forages. Also, forages do vary in their responses to fungal treatment and this influences the time at which solubles are released for fungal growth in preparation for lignocellulose degradation; some do this earlier and some later and this definitely has an effect on degradability (Akinfemi, 2010b; Mahesh and Mohini, 2013; Sharma and Arora, 2015).

The lower nutrient degradability of *P. ostreatus* pre-treated forages in comparison with *C. rivulosus* pre-treated forages might be due to higher loss of water-soluble contents from the *P. ostreatus* degraded substrates, as influenced by the PFF method, as more degraded substrates seem to contain more water-soluble contents (Arora and Sharma, 2009a), therefore leaving behind *P. ostreatus* treated substrates with higher structural carbohydrates that are capable of resisting rumen microbial attack thus reducing degradability. The results support the findings that degradability of treated substrates has been found to be influenced by the kind of substrate, fungal species, and duration of incubation (Jalc *et al.*, 1997; Arora and Sharma, 2009a; Akinfemi, 2010b), as different fungal species vary in the time they take to degrade lignocellulose, which is also dependent on the substrate.

The low NH_3-N content recorded in the fermentation liquid containing the pre-treated forages obtained from the PFF method at the early hours of *in vitro* incubation (i.e. 24h and 48h) is a reflection of the source of CP content (i.e. from chitin). CP has been found to be positively correlated to NH_3-N (Niu *et al.*, 2018). Therefore, the low NH_3-N content recorded is an indication that the absolute CP content was not increased, but the fungal biomass/chitin increased the CP content. Chitin polymers degradation in the rumen is very slow, as the polymers resist rumen microbes' degradation, which in turns lowers the concentration of NH_3-

N in the fermentation fluid (Di Mario *et al.*, 2008; Niu *et al.*, 2018). However, the reported NH_3-N increase recorded at a later hour of *in vitro* incubation (i.e. 96h) might indicate that the rumen microbes have adapted themselves to the presence of the chitin (Cobos *et al.*, 2002) and were now capable of degrading the chitin after this longer period of incubation. The lower initial NH_3-N content might be the reason why the nutrient degradability was low, as it's required in adequate concentration for an effective fibre degradability (Hoover and Stokes, 1991). The obtained results at early incubation hours were not in agreement with the findings of Omer *et al.* (2012) and (Salman *et al.*, 2008), where a higher amount of NH_3-N was recorded in the fungal treated substrates. The other reason, apart from the slow rate of chitin degradation, might be due to the loss of solubles caused by the PFF method, as proteins were perhaps part of the solubles.

The higher NH_3-N content obtained in the fermentation liquid containing the 28 days pe-treated forages compared to the 14 days pre-treated forages obtained from the PFF method might be because less solubles, which included soluble protein, were lost when they were pump filtered due to increased porosity and lower availability of solubles in the 28 days pre-treated forages. The lower soluble loss has allowed greater retention of absolute CP content, in addition to the increased CP caused by fungal biomass (Chen *et al.*, 1995; Belewu and Belewu, 2005), that was subsequently degraded by rumen microbes for a possible release of NH_3-N.

The increased NH_3-N content in the fermentation fluid of *C. rivulosus* pre-treated forages at early hours of *in vitro* incubation times (i.e. 24h and 48h, but not 96h) in comparison with *P. ostreatus* pre-treated forages can be attributed to lower loss of crude protein from the degraded forages during pump filtering, which provided proteolytic rumen microbes with more nitrogen needed for NH_3-N synthesis (Ørskov and McDonald, 1979). *P. ostreatus* was found to be producing higher growth and activity in lignocelulose degradation, and this would have been complemented with a higher increase in CP content (Belewu and Belewu, 2005; Sharma and Arora, 2010a). However, due to the PFF method, higher loss of crude protein might have been recorded than for the *C. rivulosus* treated forages, thus reducing the amount of nitrogen available for NH_3-N synthesis. However, the higher NH_3-N content recorded in the fermentation fluid containing *P. ostreatus* pre-treated forages after 96h of *in vitro* incubation might be because the incubation time increased the rumen microbes which were capable of producing more microbial protein. The increased protein content, therefore, provided enough nitrogen needed for NH_3-N synthesis by the rumen microbes. Microbial protein synthesis has been found to be positively correlated with NH_3-N concentration (Dewhurst *et al.*, 2000; Nagaraja and Titgemeyer, 2007).

The reduced CH_4 content of the total gas released by fungal pre-treated forages when fermented can be linked to less degradability of the pre-treated forages. The obtained results were in agreement with the findings of Akinfemi (2010b) and (Akinfemi *et al.*, 2009), where a reduction in CH_4 production was recorded in pre-treated substrates. However, the basis of reduction was different, in that the fungal pre-treated forages shifted the production of acetic to propionic acid in their results. This might have been the case in this study if the solubles that would have been utilised by rumen microbes were removed by the PFF method. If the pre-treated forages obtained from the FFF method, that was close to an ideal farm method of pre-treatment, were investigated for *in vitro* studies, such possible CH_4 reduction with an increase in degradability might have been recorded. The increased CH_4 content recorded in *C. rivulosus* pre-treated forages in comparison with *P. ostreatus* is a confirmation that the reduction is linked to degradability; the more degraded the pre-treated forages are, the more CH_4 is released in this study. The reduced CH_4 content of some of the 14 days pre-treated forages in comparison with the 28 days pre-treated forages can also be linked to degradability and tGP.

The tGP in this study followed the same trend as the degradability, as well as the VFAs concentration, as gas production is seen as a measure for degradability, particularly carbohydrates degradability, which is measured in terms of volatile fatty acids (Blümmel *et al.*, 1997; Cone and van Gelder, 1999; Liu *et al.*, 2002; Cone and Becker, 2012; Zuo *et al.*, 2018) when feed is incubated with rumen fluid in an *in vitro* study (Menke *et al.*, 1979). However, the reduced tGP from the 14 and 28 day pre-treated forages obtained from the PFF method compared to the untreated forages can be attributed to their chemical composition, as the loss of solubles led to the recorded high fibre and lignin contents which were unable to provide rumen microbes with the available nutrients needed for their growth and activity. This reduces the rate and extent of structural carbohydrate fermentation (Wilson and Hatfield, 1997) that is required to obtain higher forage degradability (Van Soest, 1994; Osuga *et al.*, 2006), and this directly reduced the amount of gas produced (Njidda and Nasiru, 2010). The higher tGP recorded in *A. gayanus* and *L. perenne* when pre-treated for 14 days, and the higher tGP for *B. decumbens* and *T. aestivum* when pre-treated for 28 days, can be attributed to the forage nutrient composition and time of increased availability of chitin. Forages vary in the time at which they release solubles when pre-treated with fungi (Arora and Sharma, 2009a; Sharma and Arora, 2015), and with the use of the PFF method it is expected that the time at which solubles are lost varies, indicating that the increase in the insoluble fraction caused by the PFF method will change and the negative impact on digestibility will differ as well, thus affecting the tGP at different times. The other reason might be that the fungi produced higher chitin on these

substrates at a different period of pre-treatment, as chitin tends to reduce degradability (Asiegbu *et al.*, 1995; Fadel El-Seed *et al.*, 2003; Di Mario *et al.*, 2008). The lower tGP recorded in most of the *P. ostreatus* pre-treated forages in comparison with *C. rivulosus* pre-treated ones can be attributed to their chemical composition and chitin availability. *P. ostreatus* recorded higher growth and laccase activity than *C. rivulosus* in most of the pre-treated forages, which might have led to an increase in the amount of available soluble (that contains more digestible fraction) that was eventually lost during the PFF method, and increase in chitin content. The loss of more solubles, coupled with the increased availability of chitin, might have led to the reduction in the tGP from *P. ostreatus* pre-treated forages. The obtained results were not consistent with those reported by Okano *et al.* (2005); Chumpawadee *et al.* (2007) and Akinfemi (2010b), where fungal treated substrates recorded higher tGP compared to the untreated substrates. The differences in result were caused mainly by the processing method used on the pre-treated forages in this study. If pre-treated forages obtained from the FFF method were investigated for *in vitro* studies, as it is closer to the ideal method of handling pre-treated forages on farm, it might have increased their tGP.

The recorded increase, rather than a decrease, in the pH of fermented fluid obtained from both 14 and 28 day pre-treated forages compared to the untreated forages is an indication that the forages have not been degraded properly. The reduction in pH signifies the secretion of enzymes that are involved in substrate degradation in the rumen, coupled with the secretion of organic acids by microbes that bring about the drop in pH (Hofrichter *et al.*, 1999; Gowthaman *et al.*, 2001; Ko *et al.*, 2009; Liers *et al.*, 2011; Dong *et al.*, 2013; Nayan *et al.*, 2018). Reduced pH has been found with increasing VFA synthesis and absorption (Lana *et al.*, 1998). The higher pH in the fermentation fluid of some of the *P. ostreatus* treated forages in comparison with *C. rivulosus* pre-treated ones, as well as the higher pH of some of the 28 days pre-treated forages than 14 day ones, can be linked to their degradability; i.e. the higher the degradability the less the pH (Cone *et al.*, 1997; Cone and Becker, 2012). The obtained results were in agreement with the findings of (Omer *et al.*, 2012), where ruminal pH was increased in the fungal treated substrates, but were not in agreement with the findings of Suzuki *et al.* (1995), where reduced pH was reported in the fungal treated substrates. The differences might be attributed to the availability of digestible fractions in the pre-treated forages.

The variations recorded in the TVFA of the pre-treated forages aligns with their degradability and tGP (Nayan *et al.*, 2018). However, the recorded reduction in the molar concentration of volatile fatty acids in the fermented fluid containing the 14 and 28 day pre- treated forages obtained from the PFF method is probably due to the presence of higher amounts of structural

carbohydrates, fungal chitin, and loss of solubles that contain the digestible fractions. All of these factors might have prevented the growth and activity of rumen microbes (Asiegbu *et al.*, 1995; Zadrazil *et al.*, 1999b; nee'Nigam *et al.*, 2009; Sharma and Arora, 2015) in properly degrading the lignocellulose in the pre-treated forages, from which VFAs are made available to the animals.

The recorded further reduction in VFAs in the fermented fluid containing some of *P. ostreatus* pre-treated forages in comparison with *C. rivulosus* pre-treated forages might be because *P. ostreatus* had extensively degraded the available dietary carbohydrates, which were then lost by the PFF method (Karunanandaa *et al.*, 1995; nee'Nigam *et al.*, 2009), therefore leaving behind pre-treated forages containing a high degree of crystalline substrates that cannot be adequately degraded by rumen microbes. The reduced VFAs recorded in this study were not in agreement with the findings of other researchers, where treated forages recorded higher TVFAs. However, the obtained result was similar to the unchanged TVFAs produced by treated straws as reported by Jalc *et al.* (1997), but was not similar to the reported increased acetic acid and propionic acid production by most of the fungi, except only one fungus that recorded lower acetic acid and propionic acid production (Jalc *et al.*, 1997). The differences can be attributed to the availability of degradable polysaccharides or carbohydrates in the obtained fungal-pre-treated substrates (Blümmel *et al.*, 1997; Getachew *et al.*, 1998; Cone and Becker, 2012; Zuo *et al.*, 2018). The iso-acid (i.e. isovaleric and valeric acids) concentrations were in line with the NH_3-N concentration. This supports the findings that the iso-acids were products of amino acid fermentation and also NH_3-N. This is because, during substrate fermentation in the rumen, amino acid deamination and further breakdown of the amino acids carbon skeleton led to the production of NH_3 and iso-acids (butyric, isobutyric, valeric and isovaleric acids) respectively (Hume, 1970a; Niu *et al.*, 2018).

5.5 Conclusion

The major conclusions that can be drawn from this study are as follows:

- The substrate-liquid ratio of 1:5 supported increased growth and laccase activity of the fungi, which made more solubles available. This condition, therefore, helped in achieving more soluble loss and led to the inability of the fungi to improve the nutrient composition and degradability of the pre-treated forages obtained from the PFF method.
- The shorter pre-treatment time helped in achieving a higher soluble loss in *B. decumbens* and *T. aestivum*, while longer pre-treatment helped in achieving a higher soluble loss in *L. perenne* and *A. gayanus*. This time period in each of the pre-treated forages increased the inability of the fungi to improve the nutrient composition and degradability of the pre-treated forages obtained from the PFF method.
- *P. ostreatus* exhibited more fungal growth and activity than *C. rivulosus* in most of the pre-treated forages. This beneficial attribute of *P. ostreatus* supported more soluble loss in the pre-treated forages when filtration methods were applied. This, therefore, further increased the inability of the *P. ostreatus* to improve nutrient composition and degradability of pre-treated forages obtained from the PFF method.
- The loss of solubles did negatively influence the ability of the fungi to improve nutrient composition, degradability and fermentation parameters of the pre-treated forages, as it reduced soluble fractions, increased insoluble fractions, reduced nutrient degradability, NH_3-N, VFAs and tGP; and increased CH_4 content, as well as pH.

5.6 Recommendations

- The loss of solubles through any form of pressure should be avoided during and after pre-treatment of the substrate, as it might be detrimental to the purpose of pre-treatment which is to improve nutrient composition and degradability/digestibility.
- Further studies are needed to investigate the effect of minimal nutrient loss on *in vitro* degradability and fermentation parameters by using pre-treated forages obtained from the FFF method.

Chapter 6

Isolation, Screening and Identification of Anaerobic Rumen Fungi (ARF) from Sheep Rumen Fluid as Possible Additives for Low Quality Forages

6.1 Introduction

The tropical forages investigated in previous chapters (i.e. Chapter 2 and 3) have been identified to be of low quality, indicating that they were not capable of meeting the nutrient requirements of ruminant animals for performance and production (Odenyo *et al.*, 1997; Van Saun, 2006). Therefore, there is the need to improve the nutritive value of these forages. An alternative way of improving these low-quality forages in terms of voluntary intake, nutrient utilization and feed degradability/ digestibility for increased animal health and performance is through the use of feed additives. Feed additives are capable of manipulating the rumen microbial ecosystem by increasing the number and activity of cellulolytic rumen microbes to enhance fibre degradation. This can also help reduce ruminal disorders by facilitating the development of beneficial rumen and intestinal microbes and by removing pathogens (Wallace and Newbold, 1995; Bodas *et al.*, 2008b; Elghandour *et al.*, 2015). There are several feed additives such as chemical, natural and microbial products that have been used for this purpose (Knowlton *et al.*, 2002; Jouany and Morgavi, 2007; Elghandour *et al.*, 2015; Nagpal *et al.*, 2015; Puniya *et al.*, 2015; Zeng *et al.*, 2015; Belanche *et al.*, 2016). Among these additives, the microbial additives (i.e. probiotics or directly fed microbes) look promising because they are safe to use, produce no residual effect, increase palatability (Ando *et al.*, 2005) and facilitate increased number and activity of cellulolytic microbes (Elghandour *et al.*, 2015; Nagpal *et al.*, 2015; Azzaz *et al.*, 2016).

Several microbial additives exist, which include yeast e.g. *Scheffersomyces stipites, Klyveromyces marxianus* and *Saccharomyces cerevisiae* (Wallace and Newbold, 1993; Chandel *et al.*, 2015; Puniya *et al.*, 2015), aerobic fungi e.g. *Aspergillus oryzae* (Elghandour *et al.*, 2015; Puniya *et al.*, 2015) and anaerobic rumen fungi - ARF (Barr *et al.*, 1989; Gordon and Phillips, 1989; Bernalier *et al.*, 1990; Breton *et al.*, 1990; Orpin and Joblin, 1997; Puniya *et al.*, 2014). However, ARF are mostly preferred as additives because they are more consistent in improving ruminant performance and productivity through enhanced rumen fungal number and activity, and also they are natural rumen inhabitants. The ARF are usually used in liquid or dried form as additives for low-quality forages. This is evident in the methodology used by several researchers in *in sacco* studies (Elliott *et al.*, 1987; Lee *et al.*, 2000a), *in vivo* studies (Theodorou *et al.*, 1990; Dey *et al.*, 2004; Sehgal *et al.*, 2008; Saxena *et al.*, 2010), *in vitro*

studies (Paul *et al.*, 2004; Dayananda *et al.*, 2007; Nagpal *et al.*, 2009; Shelke *et al.*, 2009), and silage production (Lee *et al.*, 2015), where ARF were applied as live cultures in liquid form by oral drenching or intra-ruminal administration, or as inoculants for ensiling. Therefore, this current study tested the isolation of ARF as live cells in liquid form before their possible use as additives for selected low quality forages.

Obviously, to obtain ARF, contents of the digestive system of mammalian herbivores and ruminants are required as they exist in this environment (Trinci *et al.*, 1994; Rezaeian *et al.*, 2005; Leis *et al.*, 2014; Callaghan *et al.*, 2015; Dagar *et al.*, 2015; Hanafy *et al.*, 2017). In this study, the rumen contents of freshly slaughtered sheep were used. In sheep rumen contents, the ARF found belong to a number of genera, i.e. *Piromyces*, *Caecomyces*, *Anaeromyces*, *Cyllamyces*, *Orpinomyces* and *Neocallimastix* (Edwards *et al.*, 2017; Ranganathan *et al.*, 2017; Hanafy *et al.*, 2018), but in terms of their occurrence more *Piromyces*, *Orpinomyces* and *Neocallimastix* were reported to be present (Bauchop, 1979a; Orpin and Munn, 1986; Orpin, 1994). These fungal genera have been found to vary in their rate and extent of lignocellulose degradation (Ho and Abdullah, 1999), as well as their preferences for different substrates.

This current study, therefore, isolated several ARF by using two agar media, i.e. Orpin's (for fungal isolation) and Merck Brewer Anaerobic (for isolating fungi but mostly used for isolating anaerobic bacteria). A series of tests were carried out to study the potential for fungal growth, initially on agar plates and then in liquid medium. These tests were also used, firstly, to identify different fungi by using the pattern of their thallus development and zoospores morphology. Secondly, to select two ARF for their use in liquid form as additives with the selected low-quality forages, as possible means of improving these forages in the subsequent experiment (Chapter 6).

6.2 Materials and Methods

6.2.1. *Source of isolates*

Rumen fluid was collected from a slaughterhouse (Linden Foods Ltd.) located at Burradon, Newcastle Upon Tyne, UK, from freshly slaughtered store lambs (Suffolk cross) that reportedly were fed grass silage-based TMR plus brassica based vegetables before slaughtering.

6.2.2 *Investigating the capacity of two media towards supporting the isolation of anaerobic fungi*

Two media were used to isolate the anaerobic fungi (i.e. Merck-Brewer Anaerobic agar medium and the Orpin medium). The Merck medium (1054520500, anaerobic agar) was purchased from Merck Millipore, while the Orpin medium was manually prepared in the laboratory. The Merck medium (g / L) 51, was measured into a 2 litre bottle and sterilized by autoclaving at 121^0C for 15 mins. The Orpin's medium contained clarified (i.e. centrifuged at 2500 rpm) rumen fluid and complex medium. The complex medium was prepared in a 2 L bottle by using 150 ml of mineral solution 1 (K_2HPO_4 (g/ l) 3.0); 150 ml of mineral solution 2 (g/l) ($(NH_4)_2SO_4$, 6.0; NaCl, 6.0; KH_2PO_4, 3.0, $MgSO_4$, 0.6; $CaCl_2$, 0.6); 150 ml of clarified rumen fluid; yeast extract (2.5 g / l); trypticase peptone (10 mg / l); sodium hydrogen carbonate (6 g / l); resazurin solution (1 ml / l of 0.1% w/v); hemin solution (2 ml / l of 0.05% hemin); cysteine hydrochloride (1 g / l); agar (20 g / l) and distilled water (up to a litre) as reported by Theodorou *et al.* (2005). The agar was added to make the Orpin's solid medium but it was not added when preparing the liquid medium. The medium was then sterilized by autoclaving at 121^0C for 15min and cooled in an incubator set at 45^0C. After cooling, Kao and Michayluk (1975) vitamin solution (0.1% v/v) purchased from Sigma Aldrich (K3129-100ml) was added as described by Bryant and Robinson (1961) and Leis *et al.* (2014). The vitamin was added to accelerate microbial growth and proliferation.

In order to facilitate better isolation of fungi, filter-sterilized antibiotic solution (Penicillin-streptomycin, Sigma Aldrich, P0781-100ml) was added to the sterile media under strict aseptic conditions using sterile syringes and needles. After addition of antibiotics, the media were poured into plates in a Class II microbiological safety cabinet (Labcaire SC-R; BS 5726: 1992 and BS EN 12469:2000). Sterility tests were conducted on the media by incubating a representative portion of each medium at 28^0C for 24h to observe any possible growth. If contamination was present, the medium was rejected and re-prepared. Once they were found

to show no microbial growth, they were considered sterile and so these were inoculated with a diluted sample of rumen fluid obtained from sheep as previously described in chapters 2-3.

6.2.3 *Media Inoculation*

The inoculation was done on both sets of sterile media plates by using 50µl of inoculum (serial dilutions of samples). The inoculum was spread on the entire agar surface using a stainless steel spreader (Sigma aldrich; 25 mm L; Z363375) according to the procedure of Lowe *et al.* (1985). The serial dilution was done using the 1 litre of anaerobic dilution solution (ADS) which contained 25 ml of mineral solution 1 (described in section 6.2.2); 25ml of mineral solution 2 (described in section 6.2.2); 1 ml of 0.1% (w/v) resazurin solution; 20ml of 5% cysteine hydrochloride; 50 ml of sodium carbonate; and 879 ml of distilled water. Approximately 9 ml of the ADS was dispensed in a pre-gassed 20ml glass vial (4pcs) to represent each dilution (10^{-2}, 10^{-3}, and 10^{-4}). Each dilution was replicated four times resulting in 16 glass vials. The diluted inocula were obtained from 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions and spread onto sterile agar plates. The plates were kept in anaerobic jars containing anaerobic atmosphere generating bags (Sigma Aldrich; 68061), and an indicator that changed from pink to white colour to represent the anaerobic environment. The jars were then kept in an incubator at a temperature of 39 ± 1°C for 21 days. During incubation, the plates in the jars were regularly visually inspected every day for the appearance of fungal colonies and mycelium diameter measurement was taken every 7 days.

6.2.4 *Fungal colony isolation procedures*

Only colonies from plates inoculated with 1 x 10^{-4} ml were isolated because those colonies were distinct and easy to identify. The isolated colonies were transferred, by cutting the edge of each identified colony from each medium using a scalpel, into a freshly prepared sterile agar plate. The medium used was similar to the medium prepared for culturing the fungi. The whole process was carried out every 14 days until a single type of a colony was obtained from each medium.

6.2.5 *Fungal identification*

Morphological identification of isolated fungal rhizoids was made by light and fluorescence microscopy (epifluorescence microscope, Nikon Eclipse Ci, CoolLED pE-300-W, Japan), based on the features of zoospores, types of sporangia and the nature of the rhizomycelia, using lactophenol cotton blue as the staining solution (Chaudhry, 2000). Identification of the fungi was made up to the genus level by thallus morphology (monocentric or polycentric), the shape of sporangia, rhizoid type (filamentous or bulbous) and the number of flagella per zoospore

(i.e. mono-flagellate or poly-flagellate). The *Neocallimastix* spp were identified by monocentric thallus and several branched rhizoids with multi-flagella zoospores (Gruninger *et al.*, 2014); the *Piromyces* spp were identified by monocentric thallus and several extensive-crisscross branched rhizoids with less flagella (i.e. uniflagellate) zoospore compared to *Neocallimastix* (Orpin, 1977); the *Orpinomyces* spp. were identified by polycentric thallus growth, with a single nucleated rhizoid having many spherical sporangia (Sridhar *et al.*, 2010); the Anaeromyces sp., were identified by polycentric thallus growth and several branched rhizoids with uniflagellated zoospore (Breton *et al.*, 1990; Fliegerova *et al.*, 2002).

6.2.6 *Screening the isolated fungi for identification of the fungi exhibiting the best growth performance and enzymatic function*

The screening of the isolated fungi for colony size (diameter) on the agar plates was done for isolates obtained from both media. The fungi obtained from the Orpin's medium that exhibited the highest colony size and represented different genera were investigated in the liquid media. However, those from the Merck's medium that showed the highest colony size were not investigated further in the liquid medium because the Merck's medium without agar wasn't available for sale. The main reason for using the Merck's medium was to ascertain if it was capable of isolating anaerobic fungi because the medium was not normally used for fungal isolation.

6.2.6.1 *Growth on solid medium*

The isolated fungi were investigated for their growth on agar plates. The isolated fungi were obtained after 4 times of sub-culturing into new plates from both media, and they were cultured in petri-dishes containing both kinds of solid media (Merck's and Orpin's). Their diameters were measured at 7d, 14d and 21days using a ruler.

6.2.6.2 *Growth in liquid medium*

Five (5) of the isolated fungi with the optimum mycelium spread on agar plates using Orpin's medium were investigated for biomass production using the liquid medium. The liquid media (i.e. broth) in the serum bottles were inoculated with 2 mycelial discs (5mm mycelium diameter) under an aseptic condition, with each of the isolates obtained from a 7 day old culture grown at 39^0C, for the measurement of fungi biomass expressed in terms of dry weight. The serum bottle contents were mixed after the designated period of 4d, 7d, 10d, 14d, and 21d to aid homogenization and then filtered through a tarred cellulose acetate membrane filter (Whatman 47mm diameter, 0.45um) using a sterile filter system, after which it was placed in

a drying oven regulated at 105^0C and left overnight. The difference between the weight of filter with dry fungal biomass weight grown in media and the weight of the filter paper was estimated as the biomass concentration. The weight of the dry filter with dry fungal biomass weight grown in media was corrected with the weight of the dry filter with media to estimate media weight sticking to the filter. The filtrate was used for laccase enzymatic activity determination.

6.2.7 *Preservation of pure cultures for further research*

Cryopreservation was used for the pure isolated biomass using screw-cap cryo vials (2ml; cryovials polar safe external threading; Sigma; ARLC-RY32-50EA). About 1 ml of the fungal biomass was transferred into the vials to which 1ml of the medium mixture (91% medium, 8% ethylene glycol and 1% antibiotics) was added under continuous flushing with CO_2 using a sterile syringe and each vial was immediately screw-capped. Firstly the cryo-cultures vials containing the pure isolates were frozen in a box filled will isopropyl alcohol at -20^0C, before they were stored at -80^0C (Leis *et al.*, 2014).

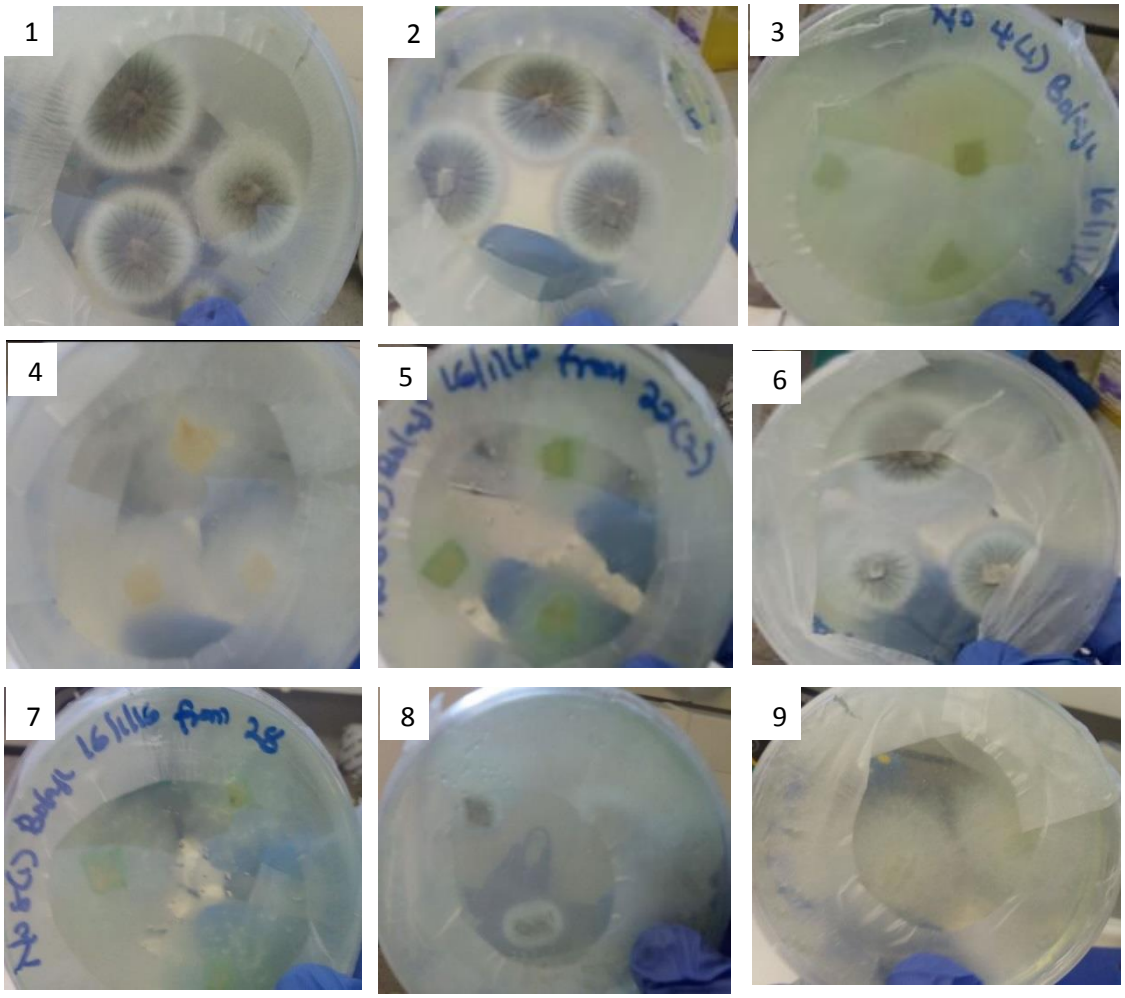
6.2.8 *Statistical analysis*

A two way ANOVA using the general linear model procedure in Minitab 16 software was used to determine the main effects of culture media and incubation times and their interactions on the growth parameters of the isolated anaerobic fungi. Tukey's test was used for the separation of differences between means for their significance at $P<0.05$.

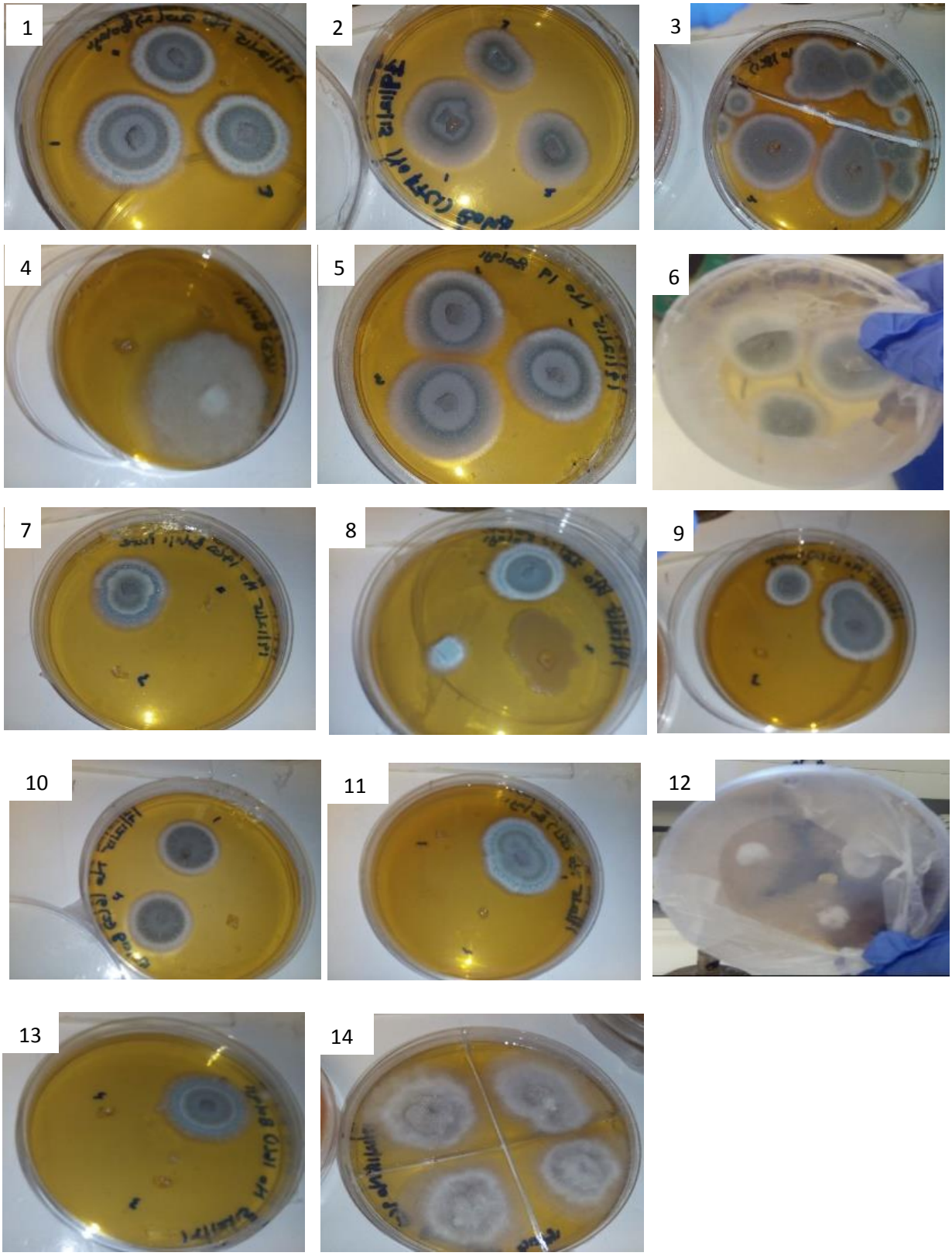
6.3 Results

6.3.1 *Isolated fungi*

The various fungi isolated from Merck's (Picture 7) and from Orpin's media (Picture 8) are shown below**.**



Picture 7 Nine (9) colonies of rumen fungi isolated using Merck's brewer anaerobic agar

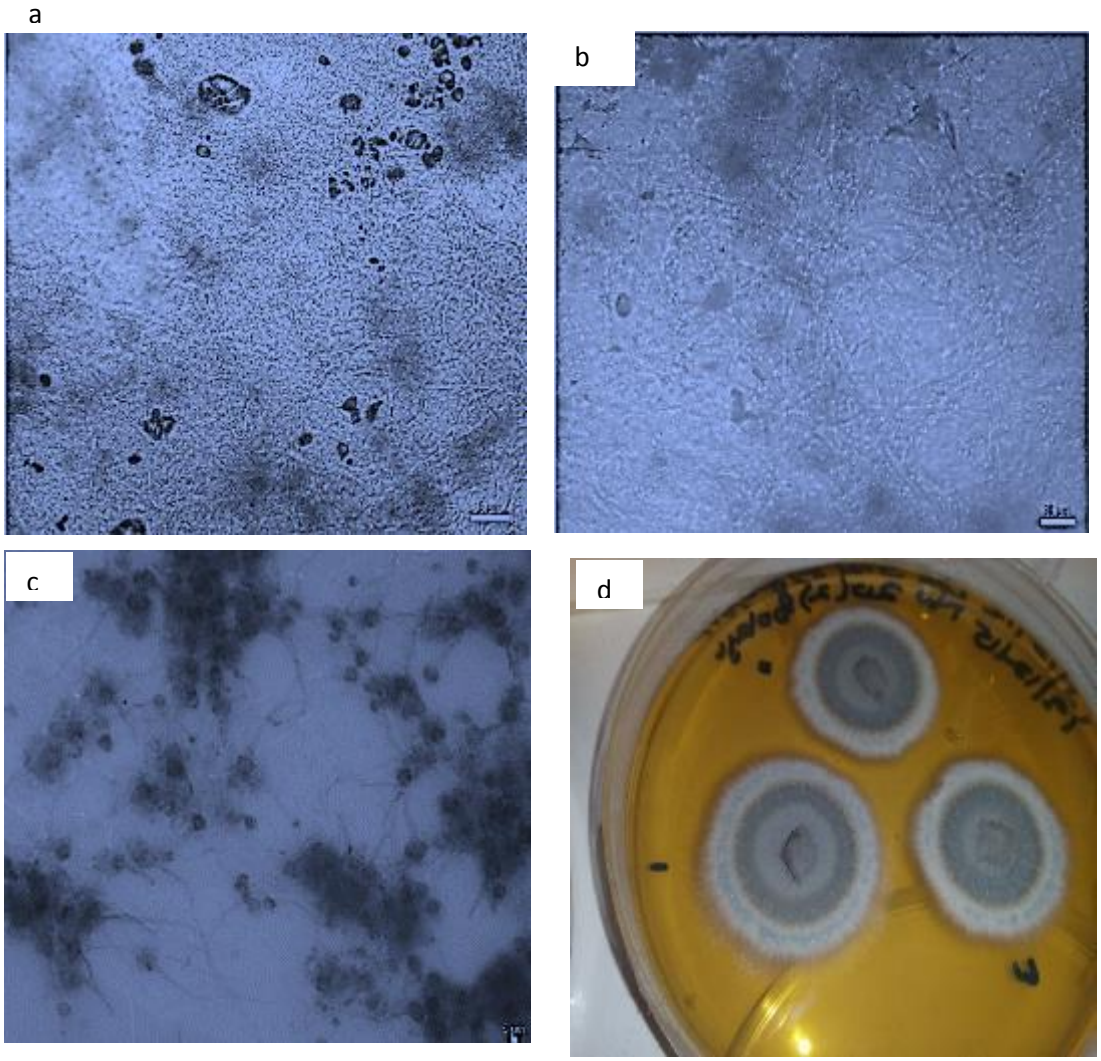


Picture 8 Fourteen (14) colonies of rumen fungi isolated using Orpin's media agar

6.3.2 *Characterization of the isolated anaerobic rumen fungi from both Merck's and Orpin's media using microscopy*

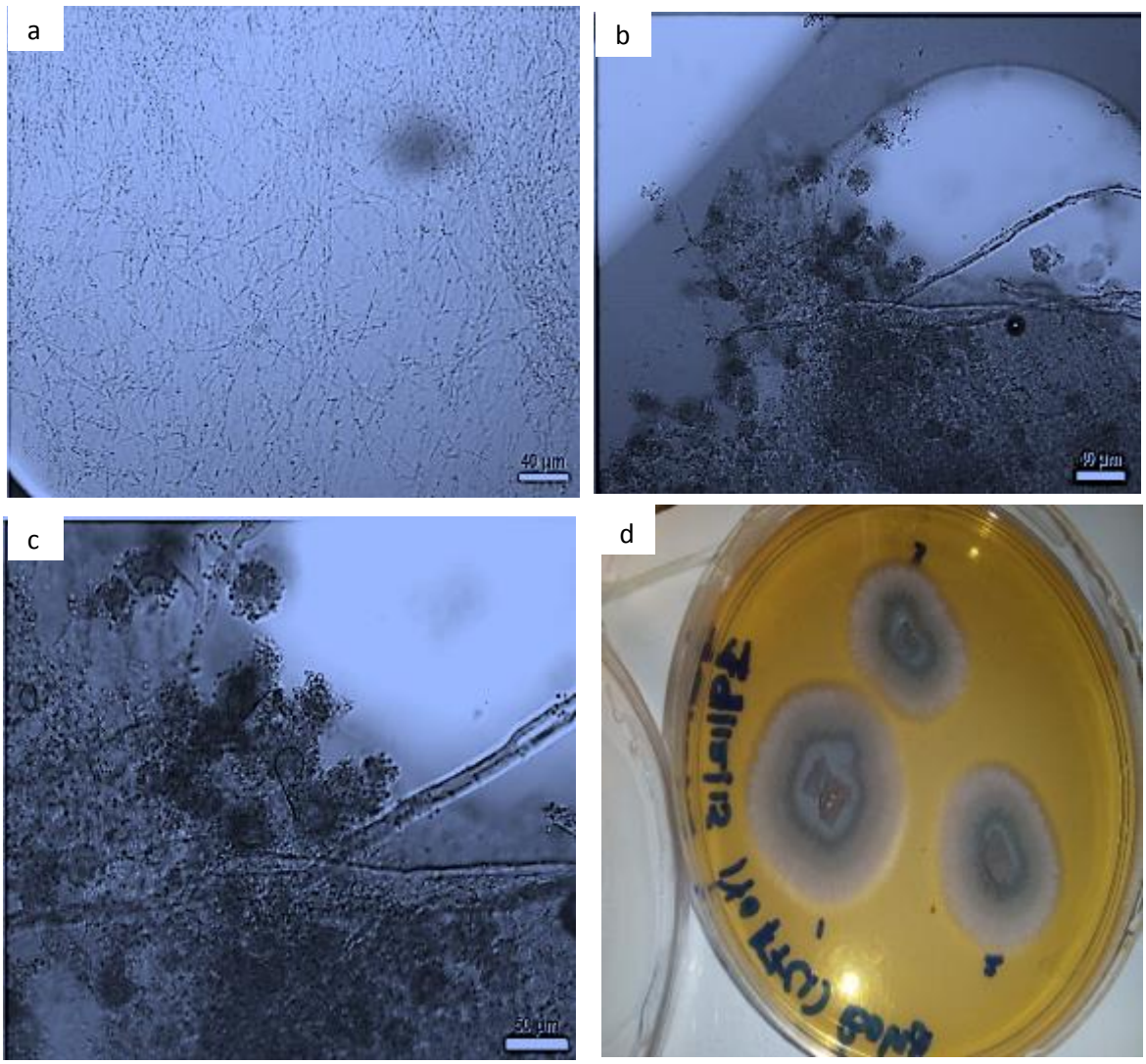
The isolated fungi from Merck's medium (Picture 7) and Orpin's medium (Picture 8) were identified under the microscope. The Merck's medium isolated fungi that belonged to the genus *Neocallimastix* (isolates 1, 2, and 6); *Piromyces* (isolates 3, 4, 5, and 8); and *Orpinomyces* (isolates 7 and 9). The Orpin's medium isolated fungi that belonged to the genus *Neocallimastix* (isolates 1, 2, 3, 5, 6, 7, 8, 9, 10, 11 and 13); *Orpinomyces* (isolate 4 and 14) and *Piromyces* (isolate 12). The pictures of the Orpin's isolates are shown because the fungi used as additives to the low quality forages (Chapter 7) were selected from these.

Isolate 1: Illustration of *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous) under various magifications



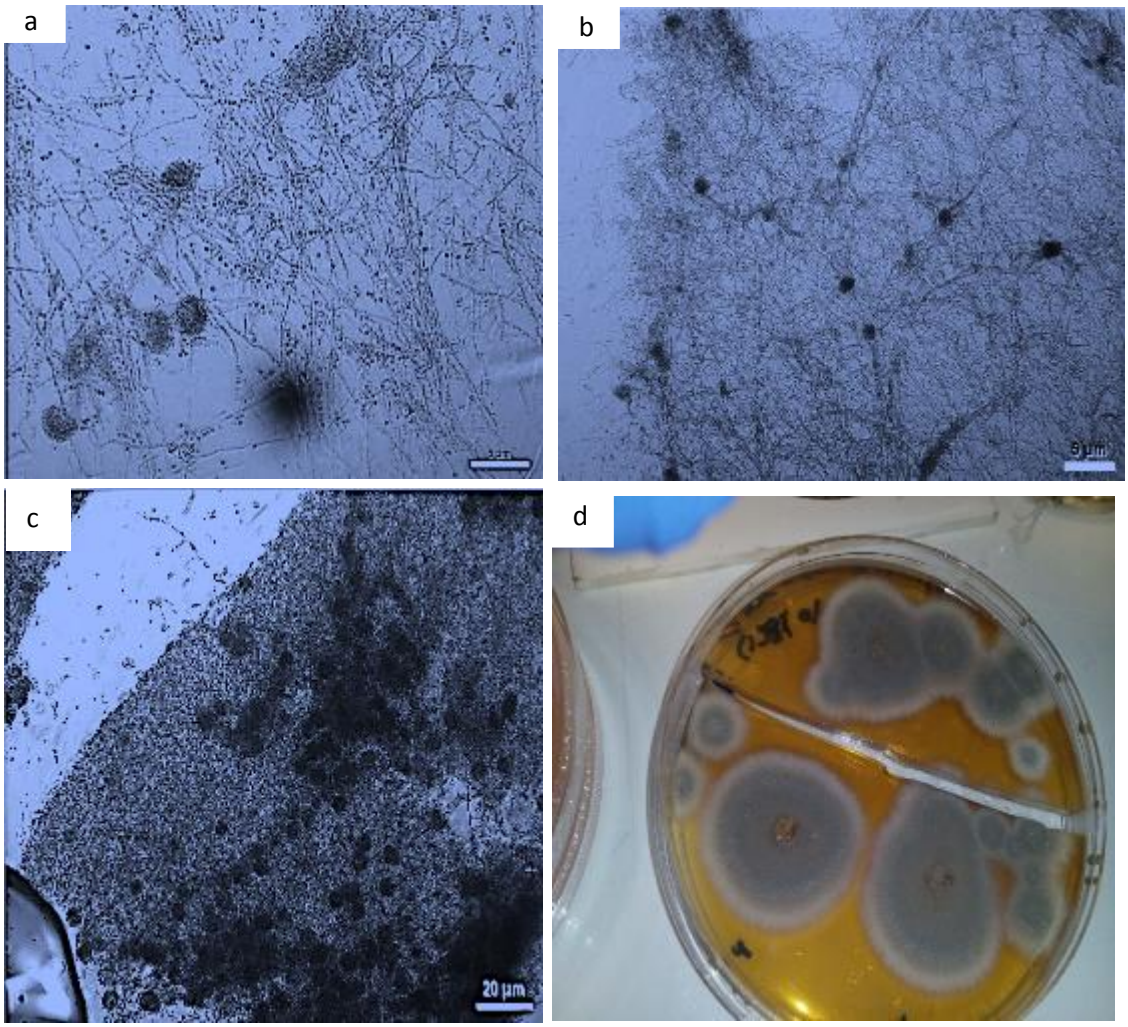
a) Hyphae of isolate 1 (20X); **b)** Hyphae of isolate 1 (40X); **c)** Spore of isolate 1 (20X) and **d)** isolate 1 on plate.

Isolate 2: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



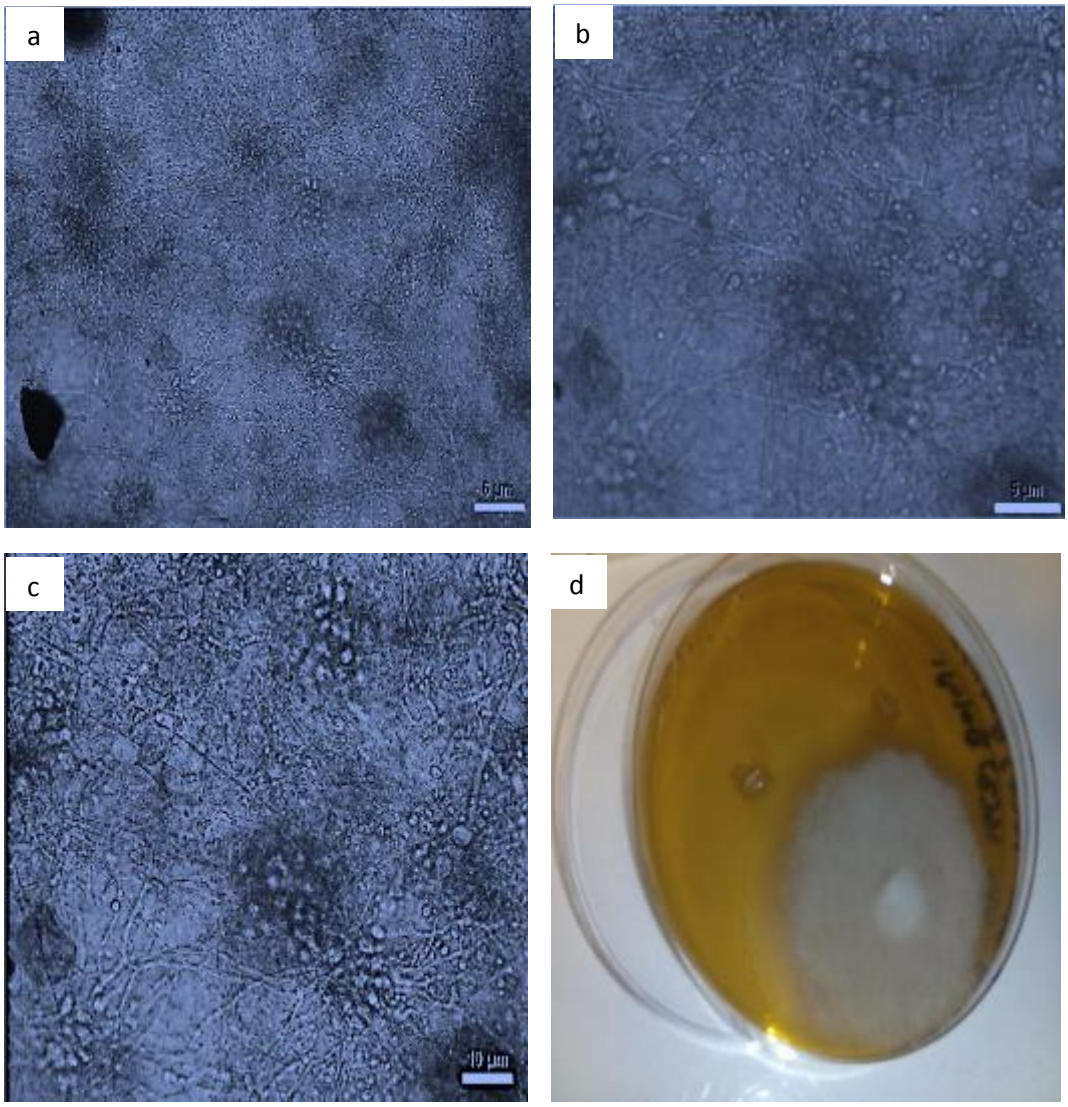
a) Hyphae of Isolate 3 (20X); **b)** Hyphae and spore of Isolate 3 (20X); **c)** Hyphae and Spore of Isolate 3 (40x) and **d)** Isolate 3 on plate.

Isolate 3: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



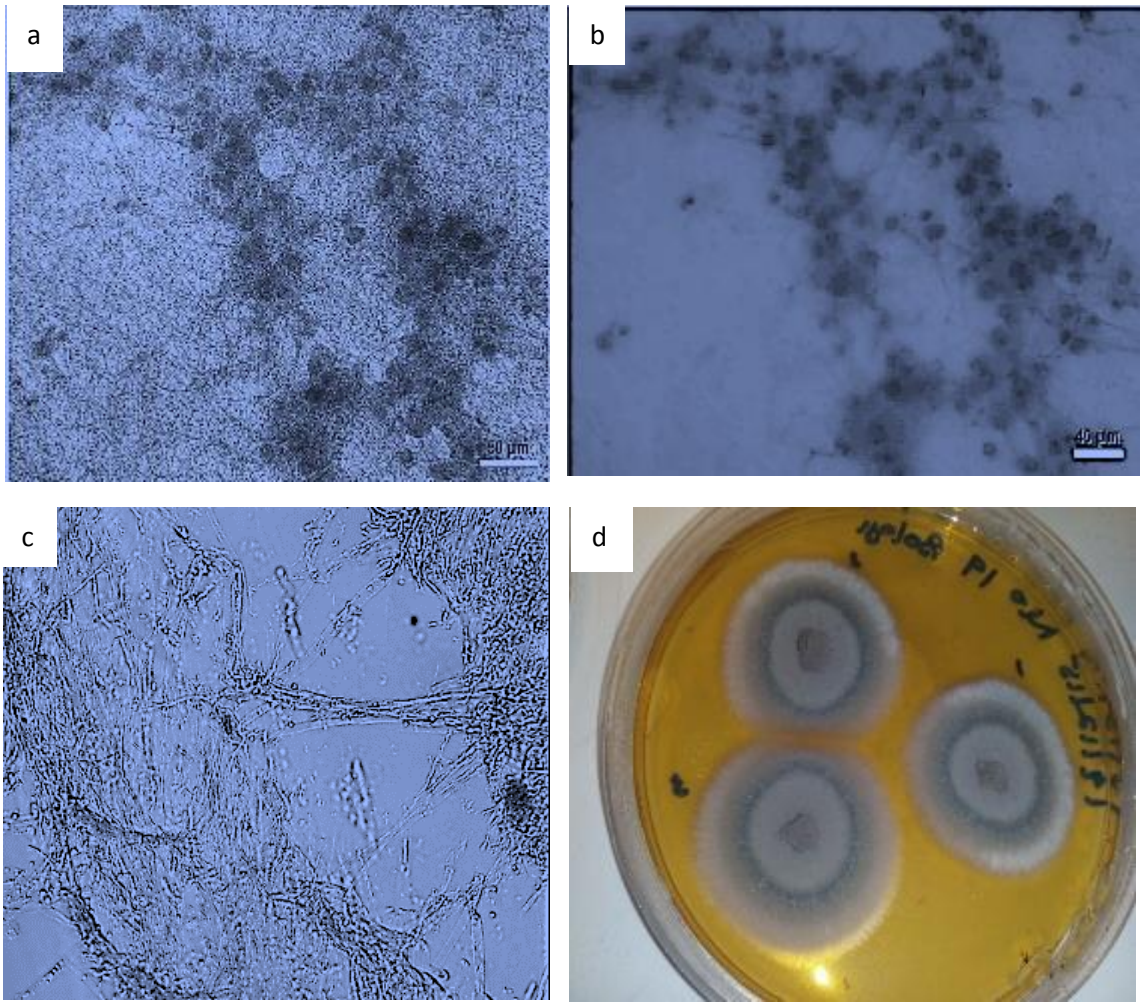
a) Hyphae and spore of isolate 2 (20X); **b)** Hyphae and spore of Isolate 2 (40X); **c)** Spore of Isolate 2 (40x) and **d)** Isolate 2 on plate.

Isolate 4: *Orpinomyces* sp (polycentric, multiflagellate, filamentous)



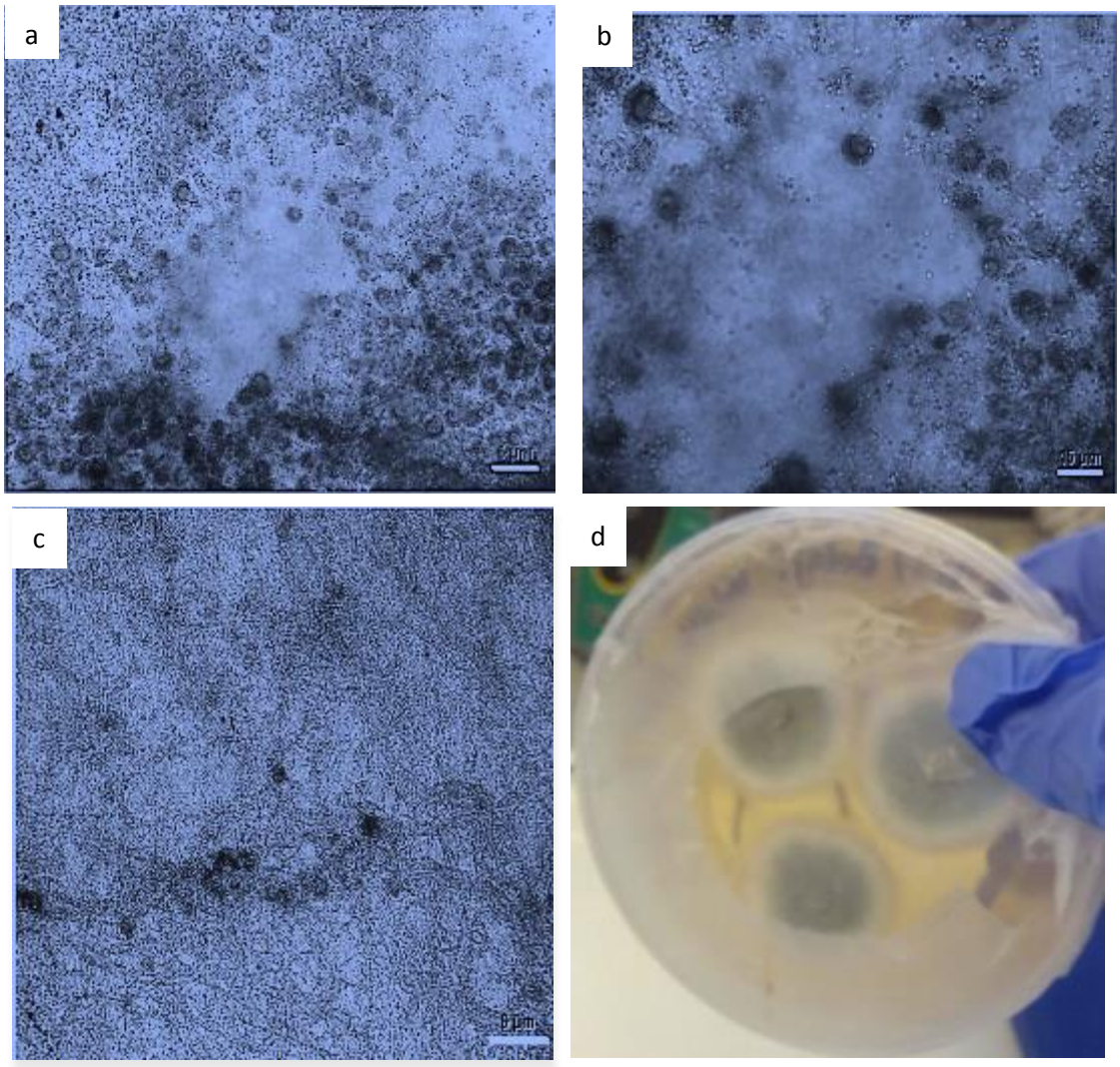
a) Hyphae and spore of Isolate 4 (20X); **b)** Hyphae and spore of Isolate 4 (40X); **c)** Hyphae and spore of Isolate 4 (40X) d) Isolate 4 on plate.

Isolate 5: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



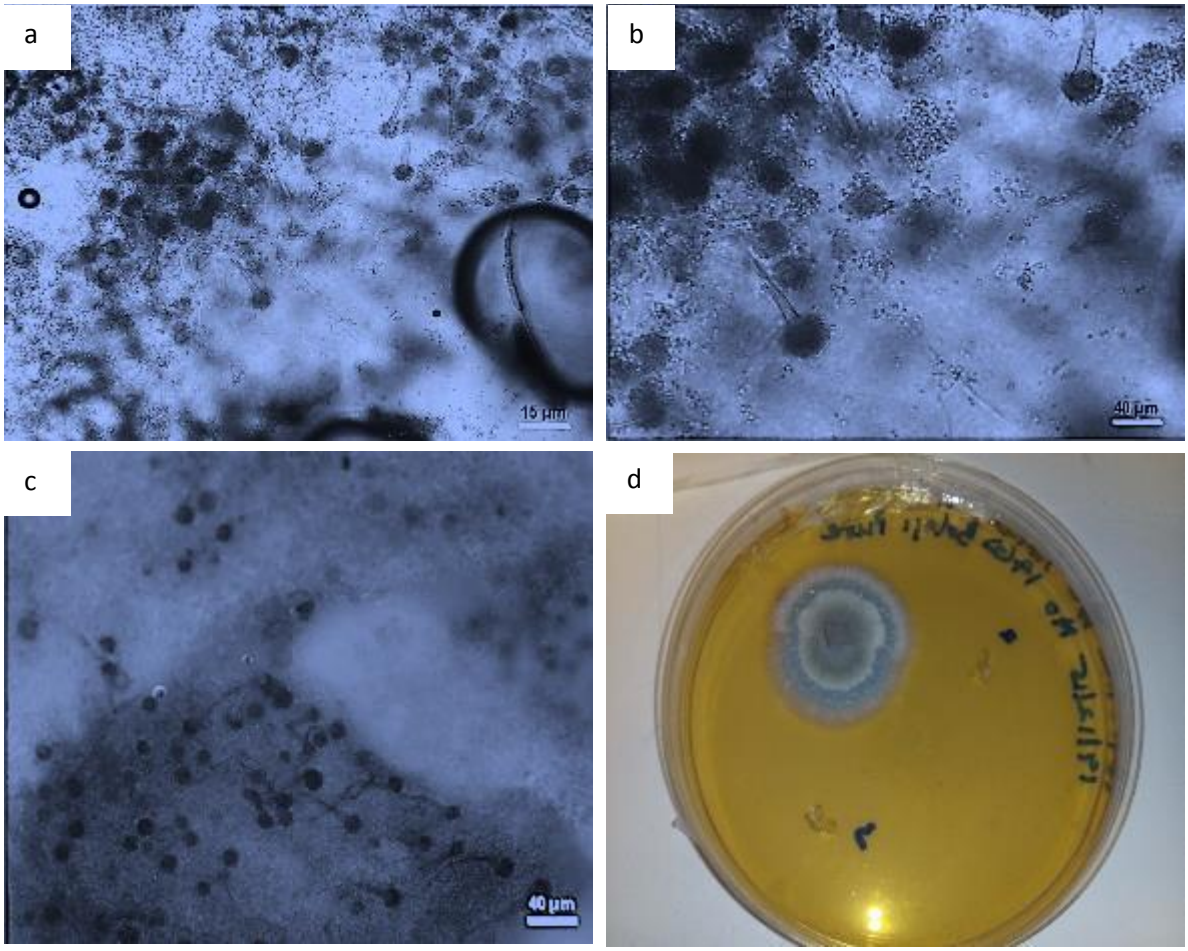
a) Hyphae and spore of Isolate 5 (20X); **b)** Hyphae and spore of Isolate 5 (20X); **c)** Hyphae of Isolate 5 (40X) and **d)** Isolate 5 on plate.

Isolate 6: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



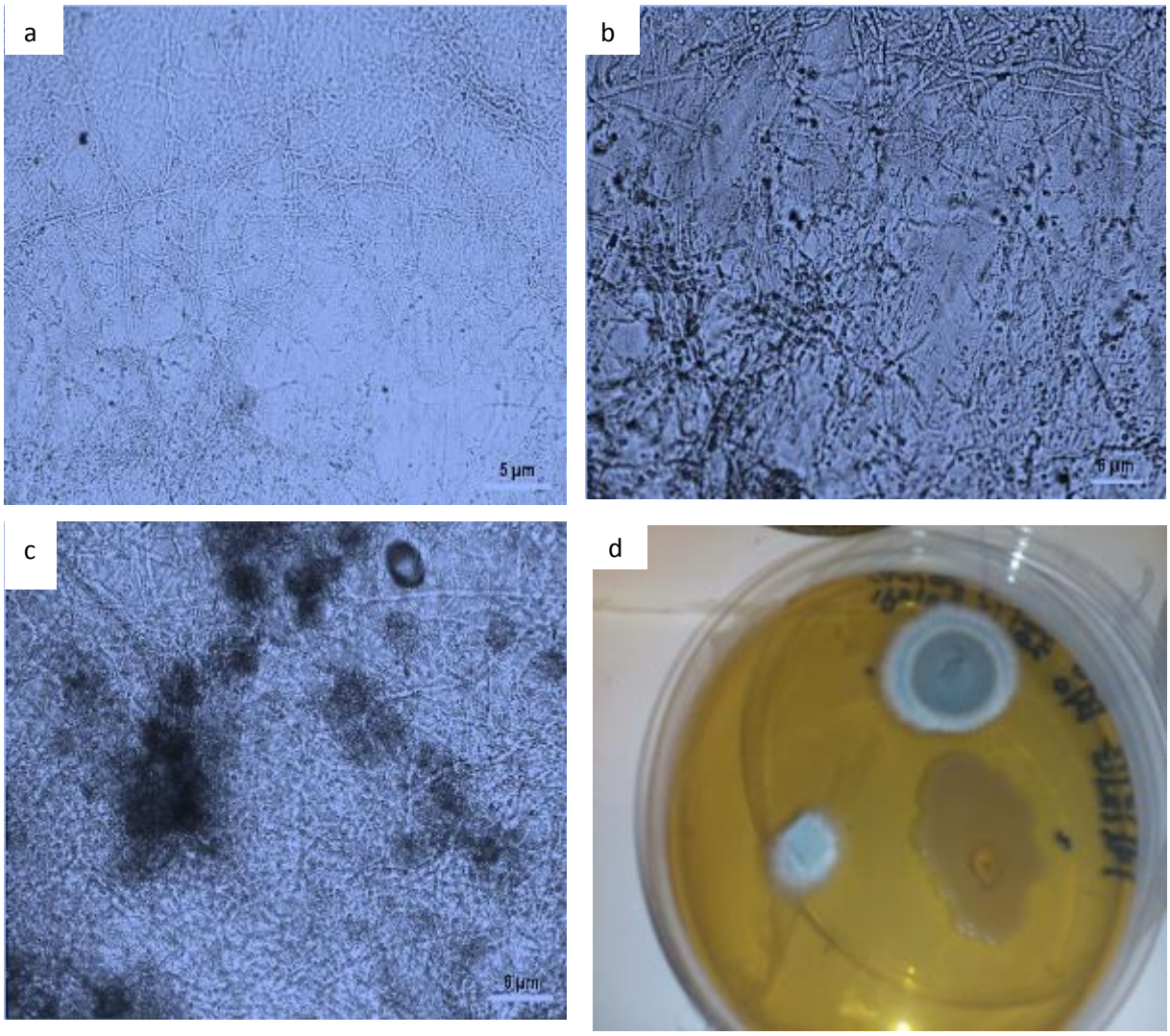
a) Hyphae and spore of Isolate 6 (20X); **b)** Hyphae and spore of Isolate 6 (40X); **c)** Hyphae of Isolate 6 (40X) and **d)** Isolate 6 on plate

Isolate 7: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



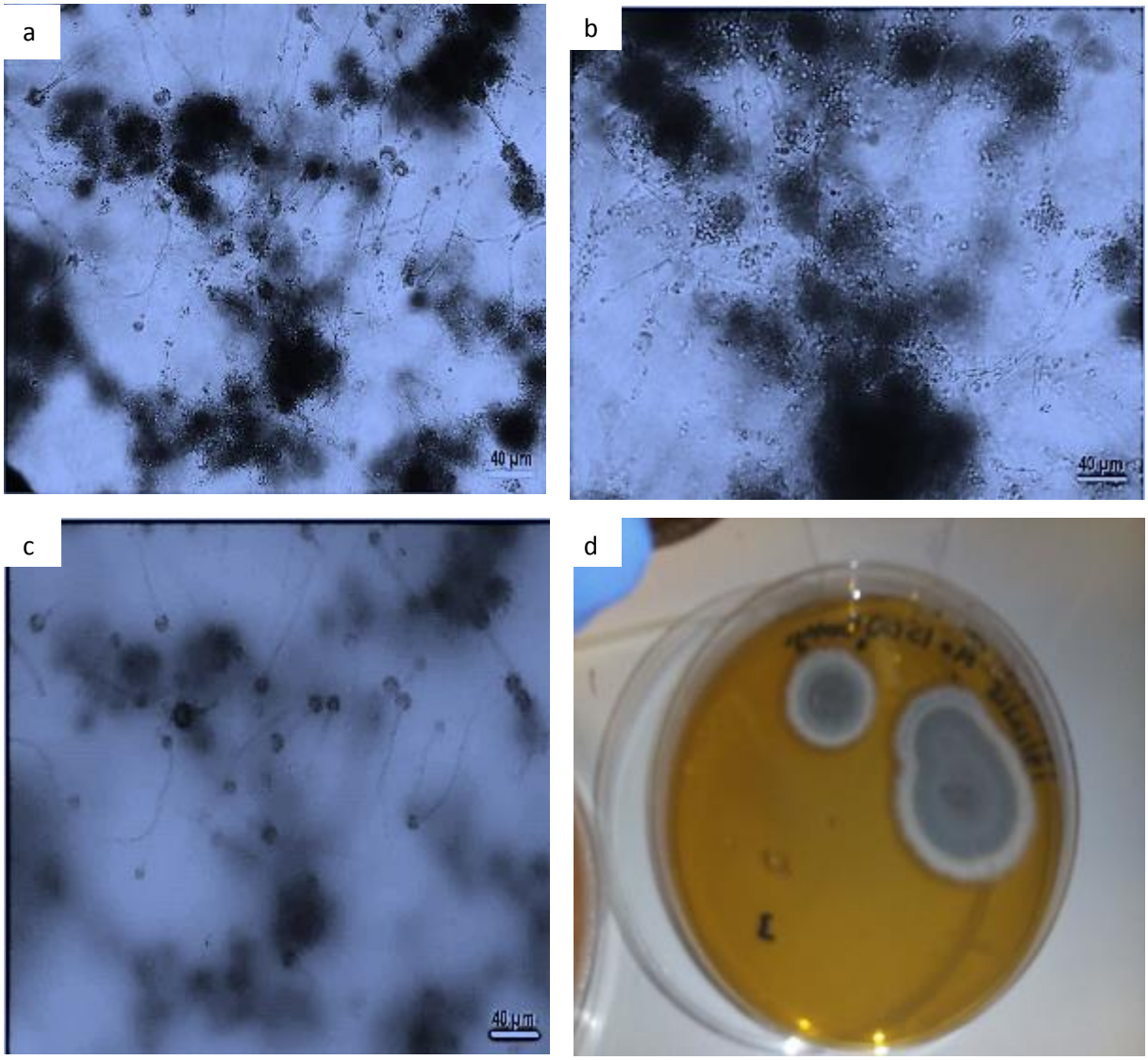
a) Hyphae and spore of Isolate 7 (20X); **b)** Hyphae and spore of Isolate 7 (40X); **c)** Hyphae of Isolate 7 (20X) and **d)** Isolate 7 on plate

Isolate 8: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



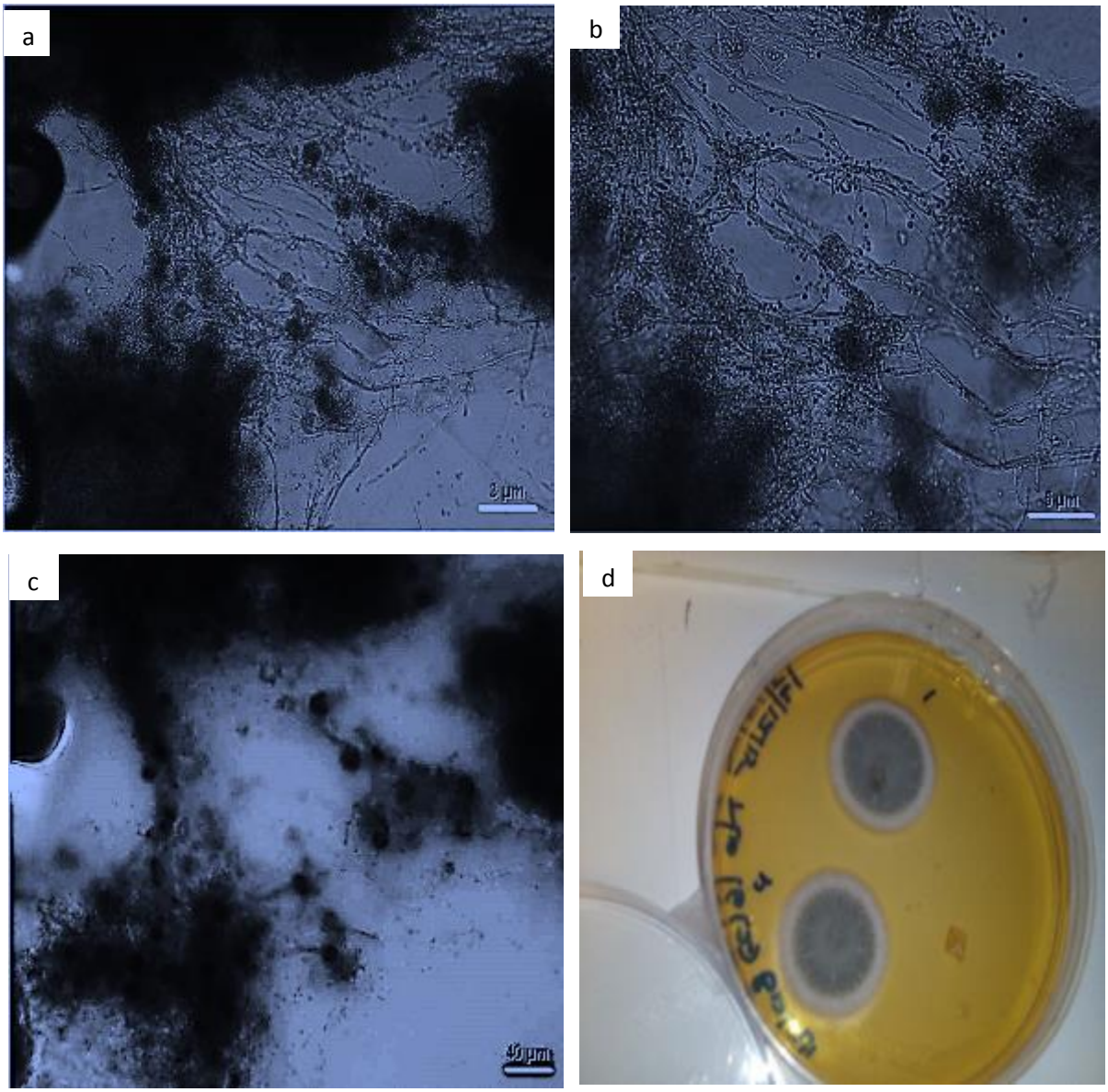
a) Hyphae of Isolate 8 (20X); **b)** Hyphae of Isolate 8 (40X); **c)** Hyphae and spore of Isolate 8 (40X) and **d)** Isolate 8 on plate

Isolate 9: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



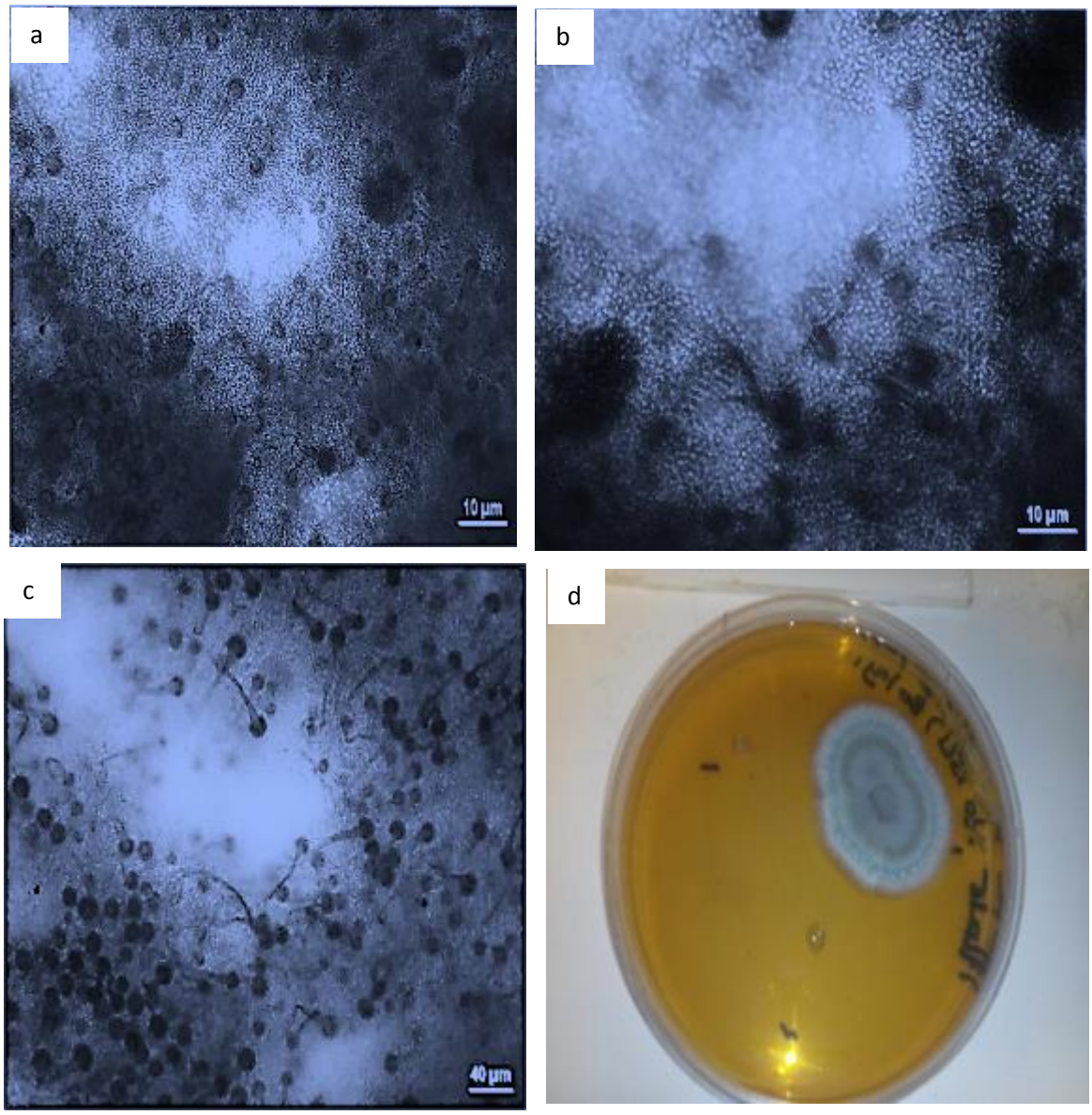
a) Hyphae of Isolate 9 (20X); **b)** Hyphae of Isolate 9 (40X); **c)** Hyphae and spore of Isolate 9 (20X) and **d)** Isolate 9 on plate

Isolate 10: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



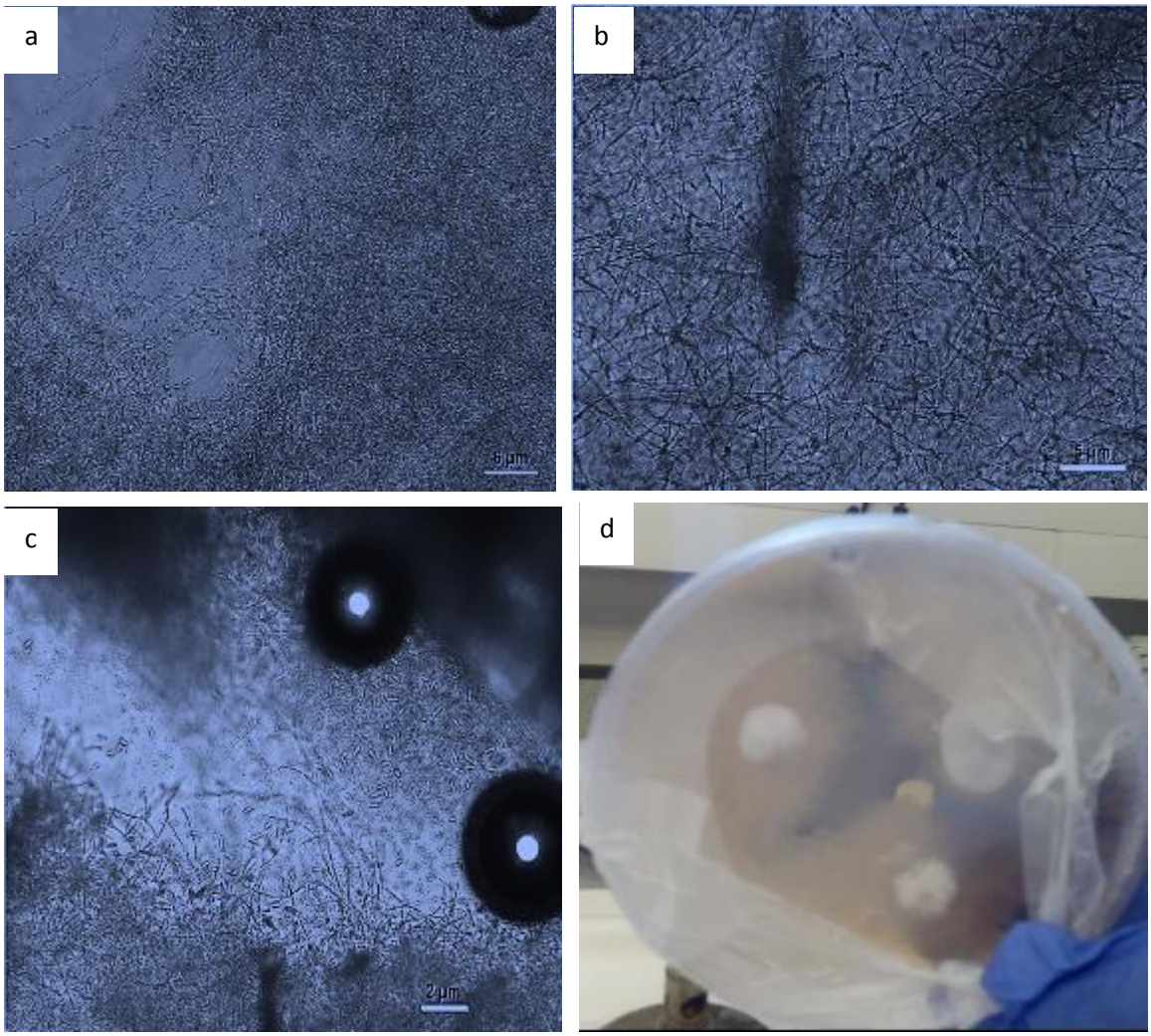
a) Hyphae and spore of Isolate 10 (20X); **b)** Hyphae and spore of Isolate 10 (40X); **c)** Hyphae and spore of Isolate 10 (20X) and **d)** Isolate 10 on plate

Isolate 11: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



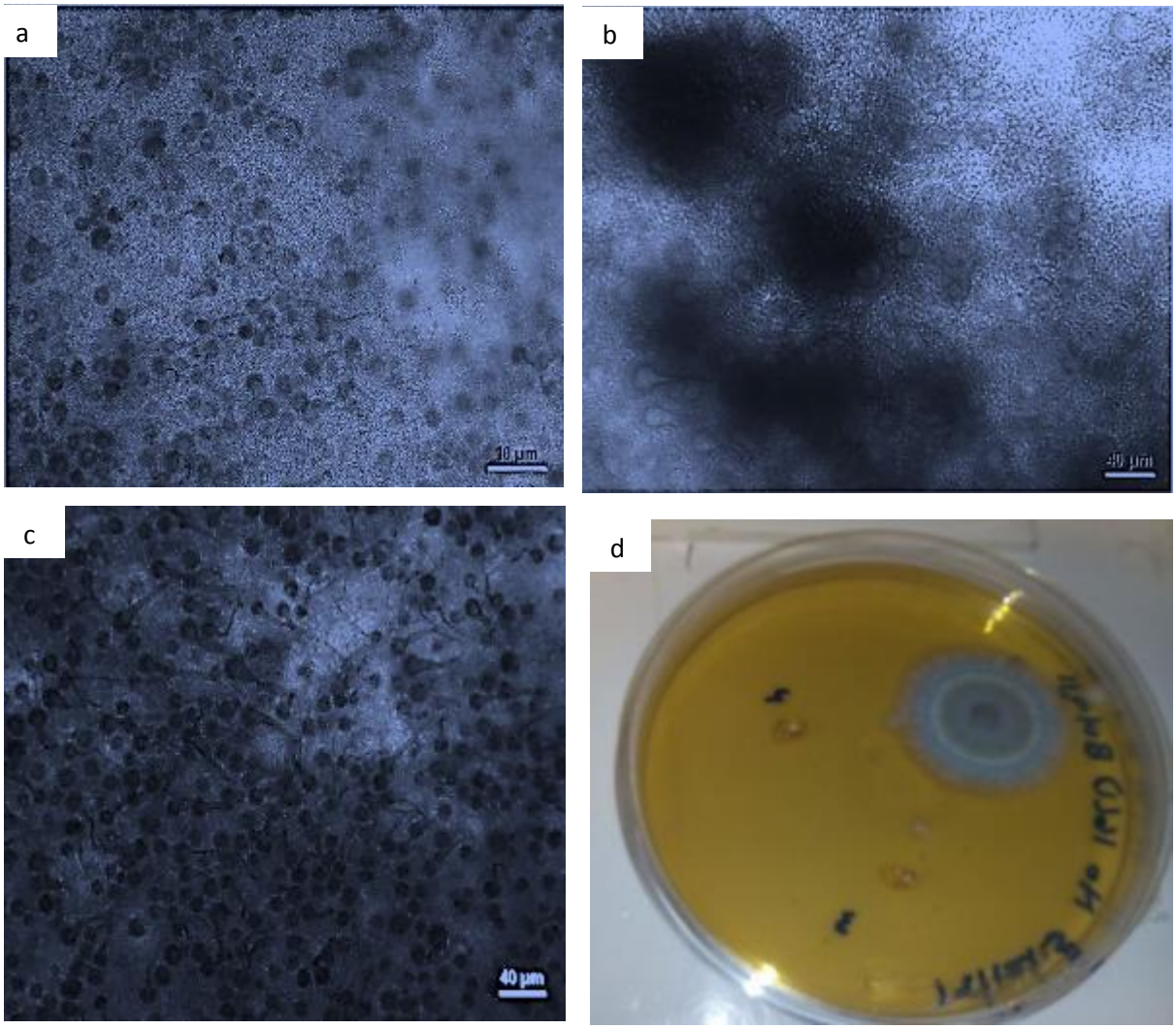
a) Hyphae and spore of Isolate 11 (20X); **b)** Hyphae and spore of Isolate 11 (40X); **c)** Hyphae and spore of Isolate 11 (20X) and **d)** Isolate 11 on plate

Isolate 12: *Anaeromyces* sp (polycentric, uniflagellate, filamentous)



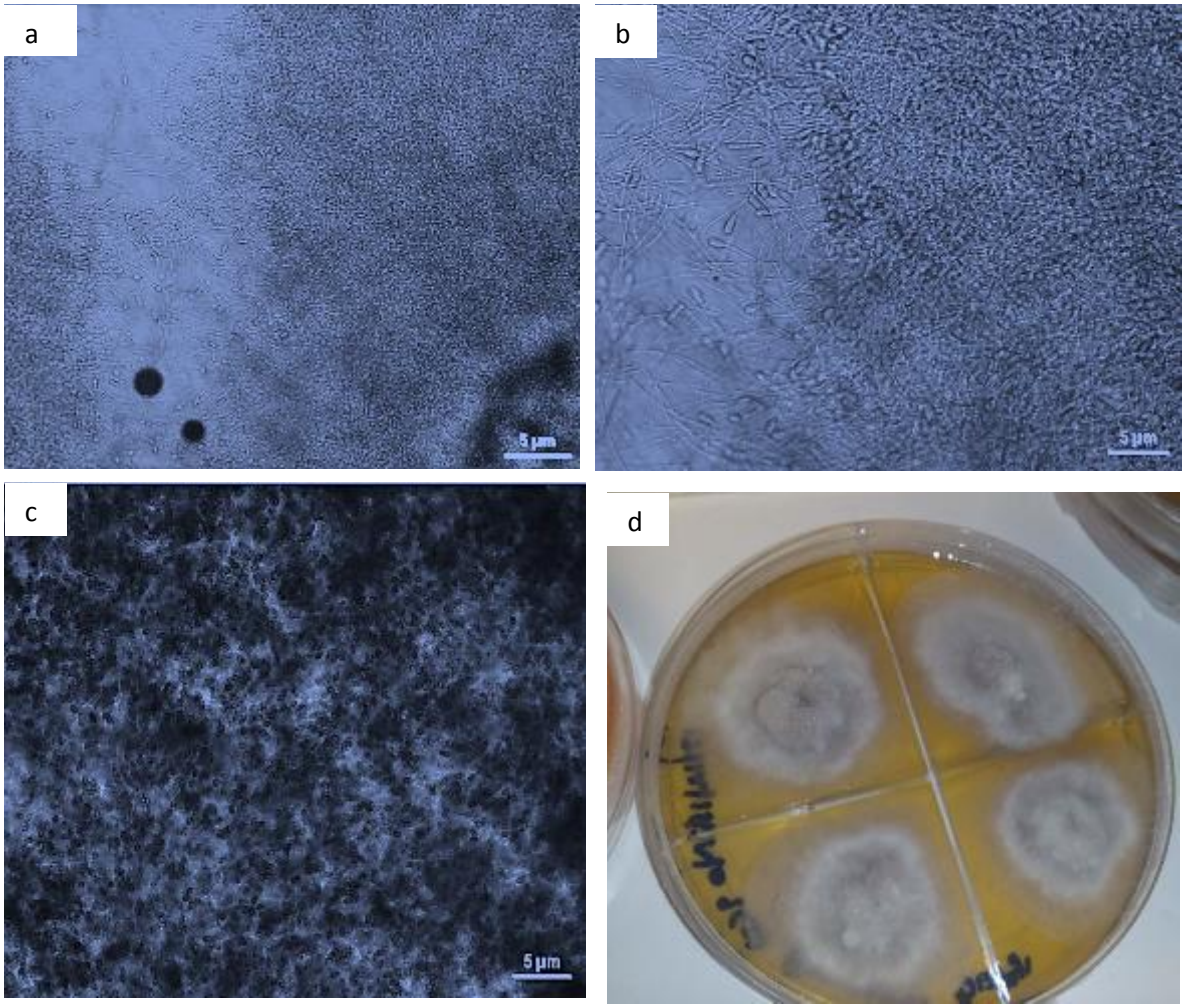
a) Hyphae and spore of Isolate 12 (20X); **b)** Hyphae and spore of Isolate 12 (40X); **c)** Hyphae and spore of Isolate 12 (20X) and **d)** Isolate 12 on plate

Isolate 13: *Neocallimaxtix* sp (monocentric, multiflagellate, filamentous)



a) Hyphae and spore of Isolate 13 (20X); **b)** Hyphae and spore of Isolate 13 (40X); **c)** Hyphae and spore of Isolate 13 (20X) and **d)** Isolate 13 on plate

Isolate 14: *Orpinomyces* sp (polycentric, multiflagellate, filamentous)



a) Hyphae and spore of Isolate 14 (20X); **b)** Hyphae and spore of Isolate 14 (40X); **c)** Spore of Isolate 14 (20X) and **d)** Isolate 14 on plate

Note: this fungus looks like *Anaeromyces* spp (Breton *et al.*, 1990) in that they are both polycentric and possess rhizomycelium but the hyphae shape was closer to the morphology of *Orpinomyces* spp, so it was classified as *Orpinomyces*

6.3.3 *Growth measurement of the isolated fungi on Orpin's solid media*

Table 66 shows the growth of each isolate obtained from sheep rumen fluid using Orpin's solid medium over 21 days of inoculation. The isolate diameters were significatly ($P < 0.05$) different from each other. The media isolated two *Orpinomyces* sp, (isolate 4 and 14) among which the highest diameter (25.0mm) was recorded; isolated one *Anaeromyces* sp (isolate 12) with a diameter similar to that of the *Orpinomyces* sp; and isolated eleven *Neocallimastix* sp (isolate 1,2,3,45,6,7,8,9,10,11,and 13) among which the lowest diameter (15.75m) was recorded. Figure 10 shows the diameter (mm) of each isolated fungi at 14 and 21 days of inoculation on Orpin's solid media. The diameter of each isolate increased as the incubation time increased. However, at 14 days of incubation, the isolates diameter was different from each other compared to the 21 days of incubation where they look similar except in isolate 11.

Table 66 Diameters (mm) of isolated fungi on Orpin's solid medium over 21 days of inoculation

Fungi isolates	Diameter (mm)
1 (*Neocallimastix* sp)	22.33^{abcd}
2 (*Neocallimastix* sp)	21.58^{cd}
3 (*Neocallimastix* sp	16.75^{e}
4 (*Orpinomyces* sp)	24.33^{abc}
5 (*Neocallimastix* sp)	22.67^{abcd}
6 (*Neocallimastix* sp)	22.79^{abcd}
7 (*Neocallimastix* sp)	21.75^{bcd}
8 (*Neocallimastix* sp)	21.17^{d}
9 (*Neocallimastix* sp)	21.08^{d}
10 (*Neocallimastix* sp)	20.00^{d}
11 (*Neocallimastix* sp)	15.75^{e}
12 (*Anaeromyces* sp)	24.79^{ab}
13 (*Neocallimastix* sp)	20.08^{d}
14 (*Orpinomyces*)	25.00^{a}
Means	21.44
SEM	0.630

Means with different letters in the same column were significantly ($P < 0.05$) different; SEM, standard error of means

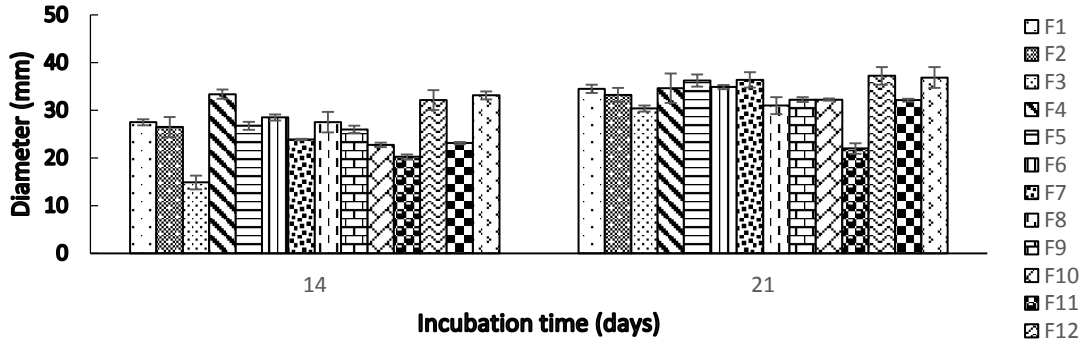


Figure 10. The diameters (mm) of isolated fungi at 14 and 21 days of inoculation on Orpin's solid medium

6.3.4 *Growth measurement of the isolated fungi on Merck anaerobic solid media*

Table 67 shows the growth of each isolate obtained from sheep rumen fluid using Merck anaerobic solid medium over 21 days of inoculation. The isolate diameters were significantly ($P < 0.05$) different from each other. The media aided the isolation of four *Piromyces* sp (isolate 3, 4, 5, and 8) among which the highest diameter (30.33 mm) was recorded; isolated three *Neocallimastix* sp, among which the lowest diameter (18.29 mm) was recorded; and isolated two *Orpinomyces* sp, among which a higher value (29.83) comparable to *Piromyces* sp was recorded.

Figure 11 shows the diameter (mm) of each isolated fungi at 14 and 21 days of inoculation on Merck's solid medium. The diameter of each isolate increased as the incubation time increased. However, at 14 and 21 days of incubation respectively, the isolate diameters were different from each other, with the isolates 9 and 4 recording the highest diameter at 21 days of inoculation.

Table 67 Diameter of isolated fungi on Merck anaerobic solid medium over 21 days of inoculation

Fungi	Diameter (mm)
1 (*Neocallimastix* sp)	21.00^{cde}
2 (*Neocallimastix* sp)	24.13^{cd}
3 (*Piromyces* sp)	25.63^{abc}
4 (*Piromyces* sp)	30.33^{a}
5 (*Piromyces* sp)	24.58^{bcd}
6 (*Neocallimastix* sp)	18.29^{e}
7 (*Orpinomyces* sp)	20.46^{cde}
8 (*Piromyces* sp)	19.50^{de}
9 (*Orpinomyces* sp)	29.83^{ab}
Means	23.75
SEM	1.209

Means with different letters in the same column were significantly (P < 0.05) different; SEM, standard error of means

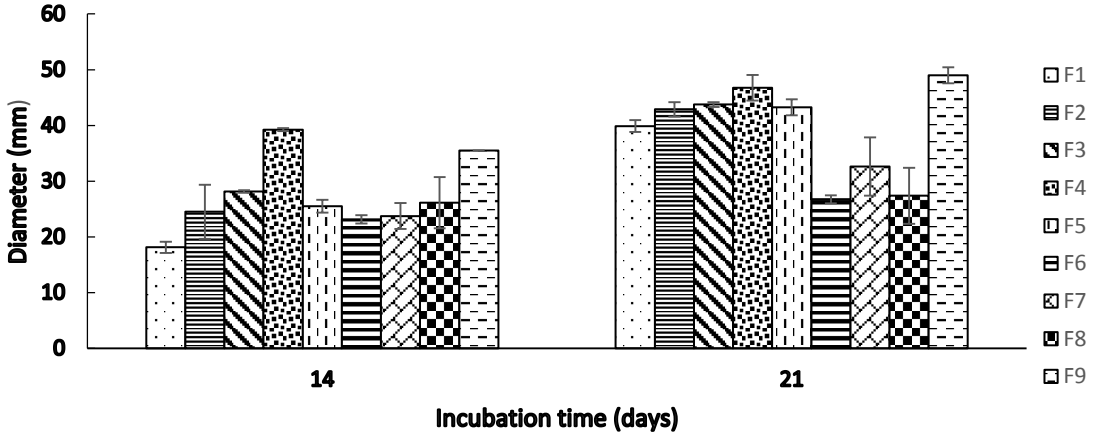


Figure 11. The diameter of isolated fungi at 14 and 21 days of inocula*tion on Merck's anaerobic solid* media

6.3.5 *Growth of the isolated fungi in liquid medium*

The effects of isolate and incubation time respectively on the dry weight (g/L) of 5 anaerobic fungi using liquid medium are shown in Table 68. The isolate and incubation time recorded significant differences for the dry weight (g/L) values. The isolates recorded the highest dry weights in following order: isolate 13 > isolate 8 > isolate 14 > isolate 12 > isolate 4. However, among individual genus, the highest dry weights followed this order: isolate 13 (*Neocallimastix* sp), followed by isolate 14 (*Orpinomyces* sp), and lastly isolate 12 (*Anaeromyces* sp). Fourteen days of incubation recorded the highest dry weight value (1.12g/ L) when compared to other incubation time.

Figure 12 showes the dry weight value (g / l) of five isolates over 21days of incubation. The isolates recorded highest significant dry weight value following the order of F8 > F13 > F12 > F14 > and F4.

Table 68 Main effect of isolate and incubation time on dry weight (g/L) values of 5 anaerobic fungi using liquid media

Isolates	Dry weight (g/L)
4 (*Orpinomyces* sp)	0.65
8 (*Neocallimastix* sp)	0.78
12 (*Anaeromyces* sp)	0.69
13 (*Neocallimastix* sp)	0.81
14 (*Orpinomyces* sp)	0.71
SEM	0.034
Incubation time (days)	
0	0.00^{e}
4	0.48^{d}
7	0.80^{c}
10	0.97^{b}
14	1.12^{a}
21	1.00^{b}
SEM	0.003

Means with different letters in the same column were significantly (P < 0.05) different; SEM, standard error of mean.

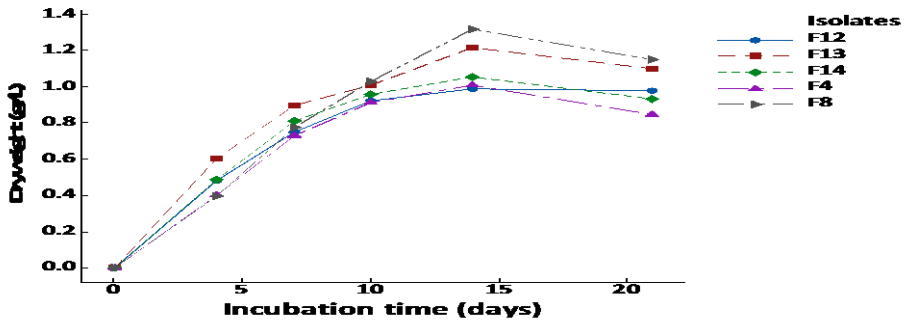


Figure 12 The dry weight (g / l) of 5 isolates obtained over 21 days of inoculation

6.3.2 *Discussion*

This study investigated the use of different media in isolating anaerobic fungi with the best growth characteristics before their use as feed additives with some selected low-quality forages. The media, i.e. Merck's anaerobic agar (a defined medium) and Orpin's (a complex medium), used in this study were capable of isolating ARF, therefore making these two media suitable for fungal isolation. The Orpin's medium has been in use in fungal isolation over many years (Orpin, 1981; Manolopoulou *et al.*, 2003; Hobson and Stewart, 2012), however Merck's anaerobic agar has not been used for fungal isolation, although it has been used for the isolation of anaerobic bacteria (Manolopoulou *et al.*, 2003; Furiga *et al.*, 2009). Therefore this study was able to identify another medium for isolating anaerobic rumen fungi apart from the known Orpin's medium. The Orpin's medium needed to be prepared, while the Merck's medium is an already defined medium. Therefore, with the identification of this medium for fungal isolation, there is the possibility that it can be used as an alternative medium, thus reducing the time involved in preparing Orpin's medium.

Identifying fungal genera with the aid of microscopic views showed that the Merck's medium supported the growth of *Piromyces* more than the growth of other fungi isolated (i.e *Orpinomyces* and *Neocallimastix*), whereas the Orpin's media supported the growth of *Neocallimastix* more than the growth of other fungi isolated (i.e *Orpinomyces* and *Anaeromyces*). This showed that different media are capable of supporting the growth of certain fungal genera more than others. Similar results were obtained by Teunissen *et al.* (1991) when different media supported the growth of different anaerobic fungi. The Orpin's medium supporting the genus *Neocallimastix* was in agreement with the findings that the *Neocallimastix* type of fungi were the most isolated fungi from ruminants such as sheep (Phillips and Gordon, 1989; Teunissen *et al.*, 1991), cattle (Orpin, 1988), and from other some herbivores such as antelope, camel and reindeer (Orpin and Joblin, 1997). However, *Neocallimastix* sp were not abundant when Merck's medium was used. This was a reflection that media composition was important in the isolation of anaerobes (Murray, 1978; Orpin, 1994; Moore *et al.*, 2011), as each of the fungal genera required different nutrients to facilitate their existence as well as growth.

The highest mycelium spread (diameter) of isolates in the Merck's anaerobic agar, as opposed to the Orpin's medium: a well-known medium for isolating anaerobic fungi, was in agreement with the findings of Teunissen *et al.* (1991) that defined media were more advantageous than complex media, in that they supported good growth of the newly isolated fungi. It also indicates

that the nutrient requirement of different fungi varied, as different media were made up of varying nutrient compositions (Murray, 1978). Isolates from Merck's medium should have been the ones to be used for further screening in liquid form. However, due to the unavailability of this medium in broth form, the fungi isolated from the Orpin's medium were the ones used for further screening of the final isolates to be used as possible additives, because thsse could be prepared in liquid form.

The growth performance on agar aided in the selection of 5 fungal isolates that had the highest mycelium spread under each genus, i.e. 2 isolates from each genus (i.e. *Orpinomyces* and *Neocallimastix)* and only (1) from the *Anaeromyces* isolates. The lowest mycelial spread recorded for the *Neocallimastix* spp on agar plates and the highest dry weight recorded in a liquid medium were an indication that those fungi exhibited different metabolic processes in different culture media (Subramaniyam and Vimala, 2012). The final screening of these isolates in broth, using the isolate with the highest dry weight in each genus and overall genera, led to the selection of the last two fungal genera, i.e. *Neocallimastix* and *Orpinomyces,* that were to be used as possible additives to the low-quality forages, with or without legume, alongside the forage controls. It is important to mention that the laccase activity of the 5 fungal isolates were determined but, because no activity was recorded, no results were displayed here. The outcome of the laccase activity measurement supported the findings that anaerobic fungi do not possess ligninolytic enzymatic function, as they have little tendency to utilise moiety for lignin when compared to the way they utilise hemicellulose and cellulose (Kirk and Farrell, 1987; Ho and Abdullah, 1999).It would have been ideal to determine their hydrolytic enzymatic function but, based on time and resources, this was not investigated.

6.4 Conclusions

The major conclusions that can be drawn from this study are as follows:

- Anaerobic rumen fungi can be isolated from both media (i.e. Merck's anaerobic medium and Orpin's medium).
- Merck's anaerobic medium supported the growth of more *Piromyces* spp., while Orpin's media supported the growth of *Neocallimastix* spp
- *Neocallimastix* spp. and *Orpinomyces* spp. were selected as possible additives to the low-quality forages in subsequent experiments of this thesis. Their use can either be as direct-fed along with the low quality forages in the rumen or as silage inoculants

Chapter 7

The effect of Anaerobic Rumen Fungal inoculation on the fermentation and Chemical Composition of selected forages

7.1 Introduction

The use of anaerobic rumen fungi (ARF) as microbial additives has received attention for improving the nutritive value of low quality forages (as discussed in chapter 6). In improving the nutritive value of forages, ARF are usually offered directly as live cultures in liquid form to ruminant animals. This research is mostly done by either oral drenching or intra ruminally *in vivo*, or in a simulated rumen environment *in vitro* (Elliott *et al.*, 1987; Lee *et al.*, 2000a; Sehgal *et al.*, 2008; Nagpal *et al.*, 2009; Shelke *et al.*, 2009; Saxena *et al.*, 2010), or less frequently as inoculants during ensiling (Lee *et al.*, 2015).

Generally, ensiling is seen as a pre-treatment method as well as a method of forage preservation (Khota *et al.*, 2016). During ensiling, the natural lactic acid bacteria that exist in the silage tend to stabilise the silage, and this is usually accompanied by fibre reduction and increase in crude protein (CP), which can be seen as an added advantage (Khota *et al.*, 2016; Pholsen *et al.*, 2016). However, with the use of feed additives, it is expected that the added advantage, i.e. fibre reduction and CP increase, is facilitated at the fermentation stage of the ensiling procedure for an improved nutritive quality alongside the preservation of silage (Lee *et al.*, 2015). Also, the use of feed additives is expected to control the microbial processes in the silage through the production of specific metabolites that inhibit the growth and activity of spoilage microbes (Weinberg and Muck, 1996; Holzer *et al.*, 2003; Neureiter *et al.*, 2005), thus preserving the silage nutrients. Once the purpose of ensiling is achieved, the obtained ensiled forage is then expected to improve digestion through enhanced microbial degradation, thus leading to an efficient nutrient utilisation by the animal.

Over the years, the most commonly used microbial additives for ensiling are the aerobic microbes, i.e. lactic acid bacteria (Nagpal *et al.*, 2015), yeast (Ando *et al.*, 2005) and aerobic fungi i.e *Aspergillus* spp (Hu *et al.*, 2011) and they exhibits high cellulolytic activity when used for ensiling. More recently, the use of ARF has received more attention, in that they exhibit higher oxidative enzymatic function (Akin and Borneman, 1990; Orpin and Joblin, 1997; Gordon and Phillips, 1998; Ho and Abdullah, 1999; Chaudhry, 2000; Rezaeian *et al.*, 2005). They possess rhizoids or mycelium which penetrates or infiltrates the plant tissues, thus aiding better substrate fermentation (Abrão *et al.*, 2014; Leis *et al.*, 2014) and increasing the accessible

sites for other microbes to colonise (Denman and McSweeney, 2006). Also, since they naturally exist in the rumen environment (Dagar *et al.*, 2011), they do not pose any palatability threat, and they show a symbiotic relationship with animals (da Silva *et al.*, 2017). These attributes make ARF more promising than other microbial additives (Puniya *et al.*, 2015) and even the aerobic fungi used in lignin degradation.. Despite all these useful attributes, the use of ARF as inoculants in silage production is still limited in improving low quality forages, as researchers are of the opinion that ARF cannot survive the pH reduction that is required to get good silage production.

However, Lee *et al.* (2015) were able to investigate the use of anaerobic fungal isolates as inoculants in rice straw silage production, and they reported that the fungal isolates facilitated the ensiling process and improved the rice straw quality. This was evident in that the fungal isolates led to a rapid reduction in pH, increased CP content, lower ammonia nitrogen content, reduced fibre content (i.e. NDF and ADF), increased fungal population, and increased *in sacco* dry matter degradability (using 30 days treated silages) compared to the un-inoculated silage. Although the reduction in pH over 120 days helped stabilise the silage, it led to a decrease in the fungal population from 30 days onwards. In their findings, the fungi were still found to be alive with an increased population for up to 30 days, even with the reduction in pH.in the rice straw silage. Also, bearing the pH reduction influence on ARF in mind, forages used in ensiling have been reported to vary in their pH reduction due to their mineral and organic acid compositions that influences their acid buffering capacity (Shaver, 1992; Muck and Kung Jr, 1997). The use of forages with high buffering capacity, and that can still support fungal growth within a short period of time, will aid in achieving better use of ARF as silage inoculants. Therefore, this current study investigated the effect of two anaerobic rumen fungal inoculants (i.e. *Neocallimastix* spp., and *Orpinomyces* spp.), isolated from sheep, on the fermentation (i.e. pH) and nutritive value (e.g. chemical composition) of silages involving two selected tropical grasses (i.e. *Andropogon gayanus*, *Brachiaria decumbens*) and two temperate forages (i.e. *Triticum aestivum* and *Lolium perenne*) as controls.

7.1.1 *Objectives*

To investigate the use of ARF (*Neocallimastix* and *Orpinomyces* spp) as inoculants on the fermentation and chemical composition of *A. gayanus* and *B. decumbens* silages, alongside the forage controls (i.e. *Triticum aestivum* and *Lolium perenne*) over 28 days.

7.1.2 *Specific objectives*

To determine the effect of anaerobic fungi as inoculants isolated from sheep rumen fluid on the silage fermentation characteristics of forages over a selected period (14 and 28 days) by evaluating the following:

- pH; proximate and fibre composition (dry matter-DM, organic matter-OM, ash, neutral detergent fibre- NDF, acid detergent fibre- ADF; secondary metabolites (total phenolics -TP, total tannins-TT) and total antioxidant capacity-TAN.

7.2. Materials and Methods

7.2.1. *Anaerobic fungi*

The two anaerobic fungi used in this experiment were isolated from sheep rumen fluid as described in chapter 6. The fungal cultures were stored with 10% glycerol in relevant 25ml serum bottles at -20^{0}C in a walk in freezer. These bottles were fitted with butyl rubber septa and sealed with metal crimps before the addition of 10% glycerol using a needle and a syringe under aseptic conditions. The cultures in bottles were thawed at 4^{0}C and then kept in a water bath at 39^{0}C for 12hr. An inoculum of 4% (vol/vol) was aseptically injected, under CO_2 atmosphere, into 25ml of the freshly prepared Orpin's medium which was then placed at 39^{0}C in an incubator - Scientific laboratory supplies incubator, INC1253 (Gordon and Phillips, 1989) for 4 - 5 days in order to get the inoculum for each of the forage samples used. A detailed description of the medium has already been provided in Chapter 6. The clarified rumen fluid (CRF) used for the medium was the same as that used in the fungal isolation, but it was used after thawing of the frozen samples as described above. After thawing, the CRF was centrifuged at 1000g for 5 min at room temperature (17^{0}C) to remove the precipitate formed during storing (Gordon and Phillips, 1989).

7.2.2. *Preparation of forage substrates and inoculation with anaerobic fungi*

The samples consisted of 4 selected forages; about 5g of each ground substrate (<2-mm particle size) was accurately weighed into 250 ml bottles and rehydrated with 10 ml of the Orpin's medium without antibiotics or vitamins. The bottles were fitted with butyl rubber caps, sealed with metal crimps, and autoclaved at 121^{0}C for 15mins. The prepared serum bottles were inoculated with 5ml of 5 days old, well grown anaerobic fungal culture in a broth by aseptically injecting the fungi into the bottles under $C0_2$ atmosphere, using a sterile syringe and a needle, and incubated at 39^{0}C for 14 and 28 days respectively. Additional flasks, each containing 5ml of autoclaved Orpin's medium and substrates but without fungal inoculations, were also prepared as controls. Eight bottles were prepared for each fungus and forage, of which four bottles were processed at the end of each incubation time along with the control flasks. The contents of each flask were filtered through tarred Whatman filter paper no. 1, and the filtrates were centrifuged at 10,000rpm (12800g) in a refrigerated (4^{o}C) centrifuge for 20 min. Each liquid part was used for the determination of enzymatic activity, while the residue on the filter paper was dried at 60^{0}C in preparation for the subsequent chemical analyses (as detailed in Chapter 5). Losses of dry matter and other nutrients as influenced by fungal inoculation were calculated as the differences in absolute weights between the respective controls (un-inoculated substrate) and inoculated substrates, and the results were presented as per cent of the control.

7.2.3. *pH and Chemical analyses*

The pH of the wet silages, as well as the chemical analyses of dried residues, were estimated as described in Chapter 2.

7.2.4. *Statistical analysis*

Two- way ANOVA using the Generalized Linear Model procedure in Minitab 16 software was used to determine the effects of fungal inoculants, forage silages and their interaction on the pH and chemical composition at each incubation time (i.e. 14 and 28 days). The effects were declared significant if $P < 0.05$. The means were separated using the Tukey's test for significance at $P<0.05$. Each value was expressed as a mean ± SE (n = 4). The model used is described as below:

$$Y_{ijk} = \mu + S_i + SL_j + E_{ijk}$$

Where:

Y_{ijk} = Observed value

μ = Population mean

S_i = Effect of ith forage

SL_j = Effect of jth fungi

E_{ijk} = Residual error

7.3 Results

The anaerobic rumen fungal inoculants had a significant reducing effect on pH values of *A. gayanus* (AGS), *B. decumbens* (BDS), *L. perenne* (LPS) *and T. aestivum* (TAS) silages during ensiling for 14 days and 28 days respectively in comparison with the control silages (Table 69). Increase in the ensiling days led to a decrease in the pH value of each forage silage, as well as their control silages, although no significant ($P > 0.05$) decrease was recorded in the pH of TAS after 28 days. The reduction in pH of each forage silage obtained from fungal inoculation was greater than the control of each forage silage. The fungi used as inoculants produced a similar reducing effect on the pH values of BDS, AGS, LPS and TAS during 14days and 28 days of ensiling. The ensiled forages varied in their pH reduction and followed this trend: LPS > tropical forages > TAS.

Table 69. Effects of anaerobic rumen fungal inoculants on pH values of each forage silage during ensiling periods for 14 and 28 days respectively.

	Main factors				
Ensiling time (days)	Control	*Neocallimastix* sp.	*Orpinomyces* sp.	SEM	P value
B. decumbens silage					
14	6.04[a]	5.80[b]	5.83[b]	0.04	<0.0002
28	5.96[a]	5.71[b]	5.71[b]	0.04	<0.0001
A. gayanus silage					
14	6.00[a]	5.91[b]	5.90[b]	0.02	<0.0005
28	5.95[a]	5.81[b]	5.74[b]	0.03	<0.0002
L. perenne silage					
14	5.37[a]	5.27[b]	5.28[b]	0.02	<0.0091
28	5.34[a]	5.19[b]	5.16[b]	0.03	<0.0003
T. aestivum silage					
14	6.28[a]	6.16[b]	6.19[b]	0.01	<0.0015
28	6.21[a]	6.11[a]	6.12[a]	0.03	<0.247
Pooled SEM	0.07	0.07	0.07		
P value	<0.0001	<0.0001	<0.0001		

Means with different letters in the same row are significantly different; SEM: standard error of means

The main effects of forage silage and fungal inoculants on the chemical composition after 14 days of inoculation are presented in Table 70. A clear effect of the anaerobic fungal inoculants on the chemical composition of the forage silages was recorded. The forages significantly ($P < 0.05$) varied in their chemical contents and secondary metabolite contents. The forage silages were high in fibre contents (i.e. NDF and ADF); low in CP except for LPS and AGS, and low in secondary metabolite contents. The fungal inoculants significantly ($P < 0.05$) reduced the forages OM, fibre contents, HEM, and TP contents, and increased the TAN contents of the silages respectively when compared with their controls. The fungal inoculants produced an almost similar effect on the chemical composition and secondary metabolite contents of the silages.

Table 70 The main effects of forage and anaerobic rumen fungi inoculants on the chemical composition (g / kg DM) of each forage silage obtained after 14 days of inoculation

	Forage silages				
Parameters	***A.gayanus***	***B.decumbens***	***L. perenne***	***T. aestivum***	**SEM**
Chemical constituents (g/kg DM)					
Dry matter (g/kg)	976.20^{b}	977.59^{b}	972.61^{b}	978.68^{a}	0.658
Organic matter	969.39^{a}	962.06^{b}	961.01^{b}	963.27^{b}	0.926
Ash	30.61^{b}	37.95^{a}	38.99^{a}	36.73^{a}	0.926
Crude protein	114.96^{b}	59.11^{c}	161.22^{a}	31.29^{d}	0.904
Neutral detergent fibre	839.31^{c}	843.79^{b}	757.07^{d}	861.74^{a}	0.388
Acid detergent fibre	578.89^{b}	574.68^{c}	544.05^{d}	588.84^{a}	0.353
Hemicellulose	260.41^{c}	269.11^{b}	213.02^{d}	272.90^{a}	0.491
Secondary metabolites (g/kg DM)					
Total phenols	8.93^{b}	8.15^{c}	10.10^{a}	6.41^{d}	0.139
Total tannins	4.96^{a}	3.97^{b}	4.80^{a}	3.11^{c}	0.118
Total antioxidants	4.14^{b}	3.85^{bc}	6.07^{a}	3.49^{c}	0.096
	Fungal inoculants				
Parameters	**Control**	***Neocallimastix***	***Orpinomyces***	**SEM**	
Chemical constituents (g/kg DM)					
DM (g/kg)	979.55^{a}	974.81^{b}	974.45^{b}	0.570	
Organic matter	968.73^{a}	961.25^{b}	961.82^{b}	0.802	
Ash	31.27^{b}	38.75^{a}	38.18^{a}	0.802	
Crude protein	90.36^{a}	91.97^{a}	92.60^{a}	0.783	
Neutral Detergent Fibre	833.50^{a}	821.43^{b}	821.50^{b}	0.336	
Acid detergent fibre	576.02^{a}	569.40^{b}	569.43^{b}	0.306	
Hemicellulose	257.48^{a}	252.03^{b}	252.07^{b}	0.425	
Secondary metabolites (g / kg DM)					
Total phenols	8.69^{a}	8.40^{ab}	8.10^{b}	0.120	
Total tannins	4.25	4.26	4.13	0.102	
Total antioxidant	4.68^{b}	3.30^{c}	5.19^{a}	0.083	

Means with different letters in the same row are significantly ($P < 0.05$) different; SEM: standard error of means

The interactive effects of forage silage and fungal inoculants produced a significant ($P < 0.05$) effect on the chemical composition of the silages after 14 days of inoculation (Table 71). The fungal inoculants reduced the DM, OM, NDF, ADF, and hemicelluloses contents, but increased the CP and ash contents. However, the increase in the CP content of each of the silages was not different from their control. *Neocallimastix* sp. reduced the OM, fibre, and hemicellulose contents, as well as increasing the CP content of the tropical forage silage i.e. AGS and BDS more than *Orpinomyces* sp., while the opposite was recorded in temperate forage silage i.e. LPS and TAS respectively. The fungal inoculants varied in their responses in the secondary metabolite contents of each of the silages when compared with the control. *Orpinomyces* sp. inoculation led to an increase in AGS secondary metabolite contents and TAN. Both fungal inoculations led to an increase in LPS secondary metabolite contents and TAN, while both fungal inoculations led to a reduction in BDS and TAS secondary metabolite contents and TAN.

Table 71 The interactive effects of forage silage and anaerobic fungal inoculant on the chemical composition (g / kg DM) and secondary metabolites (g / kg DM) after 14 days of inoculation.

		Chemical contents (g / kg DM)				Fibre contents (g / kg DM)			Metabolites (g/ kg DM)		
Forage silage	**Fungal inoculant**	**DM**	**OM**	**Ash**	**CP**	**NDF**	**ADF**	**HEM**	**TP**	**TT**	**TAN**
AG	Control	979.18^{abc}	972.71^{a}	27.29^{d}	114.04^{b}	846.18^{d}	581.63^{c}	264.54^{c}	8.97^{b}	4.75^{a}	4.20^{cd}
	Neocallimastix	974.23^{cde}	966.52^{abc}	33.48^{bcd}	116.33^{b}	835.12^{f}	577.97^{d}	257.15^{e}	8.82^{b}	4.93^{a}	2.86^{ef}
	Orpinomyces	975.17^{cde}	968.95^{ab}	31.05^{cd}	114.51^{b}	836.62^{f}	577.08^{d}	259.54^{de}	9.00^{b}	5.20^{a}	5.36^{b}
BD	Control	982.48^{a}	969.10^{ab}	30.91^{cd}	57.68^{c}	853.05^{c}	579.25^{cd}	273.80^{ab}	9.61^{b}	4.66^{a}	4.53^{c}
	Neocallimastix	976.22^{bcde}	955.68^{d}	44.32^{a}	61.04^{c}	837.17^{f}	573.46^{e}	263.71^{cd}	7.51^{c}	3.67^{bc}	2.81^{ef}
	Orpinomyces	974.08^{cde}	961.39^{bcd}	38.61^{abc}	58.60^{c}	841.16^{e}	571.33^{e}	269.82^{b}	7.32^{cd}	3.58^{bc}	4.20^{cd}
LP	Control	975.16^{cde}	967.52^{abc}	32.48^{bcd}	158.74^{a}	768.96^{g}	552.08^{f}	216.87^{f}	9.73^{b}	4.43^{ab}	6.40^{a}
	Neocallimastix	970.90^{e}	959.65^{cd}	40.35^{ab}	158.79^{a}	753.27^{h}	539.03^{g}	214.24^{f}	10.91^{a}	5.28^{a}	4.98^{bc}
	Orpinomyces	971.76^{de}	955.88^{d}	44.12^{ab}	166.15^{a}	748.98^{h}	541.03^{g}	207.95^{g}	9.65^{b}	4.70^{a}	6.82^{a}
TA	Control	981.38^{ab}	965.58^{abc}	34.17^{cde}	30.99^{d}	865.80^{a}	591.11^{a}	274.69^{a}	6.45^{cd}	3.16^{c}	3.60^{de}
	Neocallimastix	977.87^{abc}	963.17^{bcd}	36.84^{abc}	31.74^{d}	860.17^{b}	587.13^{b}	273.04^{ab}	6.35^{d}	3.15^{c}	2.53^{f}
	Orpinomyces	976.78^{abcd}	961.05^{bcd}	38.95^{abc}	31.14^{d}	859.25^{b}	588.29^{ab}	270.96^{ab}	6.44^{cd}	3.03^{c}	4.35^{cd}
Pooled SEM		1.14	1.604	1.604	1.566	0.672	0.611	0.85	0.24	0.204	0.167

Means with different letters in the same column are significantly ($P < 0.05$) different. AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent fibre, ADF; acid detergent fibre, HEM; hemicellulose, TP; total phenols, TT; total tannins, TAN; total antioxidant, SEM; standard error of means

Forage silages with anaerobic fungal inoculants recorded significant ($P < 0.05$) losses (%) in DM, OM, NDF, and consequently hemicellulose, as well as an insignificant ($P < 0.05$) CP gain and significant ($P < 0.05$) ash gain after 14 days of inoculation (Table 72). An irregular pattern was recorded in the secondary metabolite contents, in that innoculation either led to a gross significant ($P < 0.05$) loss or gain in the TP, TT and TA of the forages. In general, the fungal inoculants led to minimal losses (%) in nutrients, i.e. DM, OM, NDF, ADF and Hem, higher ash gain, minimal CP gain in each of the forage silages. *Neocallimastix* sp. recorded more nutrient losses and more CP and ash gains in AGS and BDS than *Orpinomyces* sp., while the opposite was recorded in LPS and TAS. The fungal inoculants led to minimal metabolite losses (%) in AGS, LPS, and TAS but more metabolite losses (%) in BDS, with an exception in the metabolites gain recorded in *Orpinomyces* sp. treated AGS, and *Neocallimastix* sp. treated LPS. *Neocallimastix* sp. led to TAN losses in all the forage silages, while *Orpinomyces* sp. led to TAN gain in most of the forages except in BD.

Table 72 The effects of anaerobic fungal inoculants on the nutrient loss (+) / gain (-) % of various forage silages after 14 days of incubation compared to control

		Chemical content loss / gain (%)				Fibre content loss / gain (%)			Metabolites loss / gain (%)		
Forage silages	**Fungal inoculants**	**DM**	**OM**	**Ash**	**CP**	**NDF**	**ADF**	**HEM**	**TP**	**TT**	**TAN**
A. gayanus	*Neocallimastix*	0.51	0.64^{abc}	-22.72^{abc}	-2.01	1.31^{c}	0.63^{cd}	2.79^{bc}	1.68^{b}	-3.79^{cd}	31.91^{a}
	Orpinomyces	0.41	0.39^{bc}	-13.79^{ab}	-0.41	1.13^{cd}	0.78^{cd}	1.89^{cd}	-0.38^{bc}	-9.53^{cd}	-27.88^{e}
B.decumbens	*Neocallimastix*	0.64	1.38^{a}	-43.40^{c}	-5.82	1.86^{b}	1.00^{bc}	3.69^{ab}	21.83^{a}	21.30^{ab}	37.97^{a}
	Orpinomyces	0.86	0.80^{abc}	-24.94^{abc}	-1.6	1.40^{c}	1.37^{b}	1.45^{de}	23.88^{a}	23.27^{a}	7.25^{bc}
L. perenne	*Neocallimastix*	0.44	0.81^{abc}	-24.23^{abc}	-0.03	2.04^{b}	2.36^{a}	1.21^{de}	-12.14^{c}	-19.16^{d}	22.14^{ab}
	Orpinomyces	0.35	1.20^{ab}	-35.83^{bc}	-4.67	2.60^{a}	2.00^{a}	4.12^{a}	0.82^{bc}	-6.12^{cd}	-6.61^{cd}
T. aestivum	*Neocallimastix*	0.36	0.25^{c}	-7.02^{a}	-2.42	0.65^{e}	0.67^{cd}	0.60^{e}	1.59^{bc}	0.31^{bcd}	29.56^{a}
	Orpinomyces	0.47	0.47^{bc}	-13.16^{ab}	-0.47	0.76^{de}	0.48^{d}	1.36^{de}	0.15^{bc}	4.15^{abc}	-20.98^{de}
SEM		0.1	0.2	5.6	1.3	0.1	0.1	0.3	2.9	4.9	3.8

Means with different letters in the same column are significantly ($P < 0.05$) different. DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent fibre, ADF; acid detergent fibre, HEM; hemicellulose, TP; total phenols, TT; total tannins, TAN; total antioxidant, SEM; standard error of means.

The main effects of forages and fungi on the chemical composition after 28 days of incubation are presented in Table 73. An apparent effect of the anaerobic fungal inoculants on the chemical composition of the forage silages was recorded. The forages significantly ($P < 0.05$) varied in their chemical contents and secondary metabolites contents. The forage silages seem to be high in fibre contents (i.e. NDF and ADF), and low in CP, except for LPS and AGS, and secondary metabolite contents. The fungal inoculants significantly ($P < 0.05$) reduced the OM, fibre contents, HEM, and TP contents, and increased the TAN contents of the silages respectively when compared with their controls. The fungal inoculants produced an almost similar effect on the chemical composition and secondary metabolite contents of each of the forage silages. *Neocallimastix* sp. reduced the metabolite contents of the silages, while *Orpinomyces* sp. increased the contents, although the increase in metabolite contents was not different from that recorded in the un-inoculated samples. *Orpinomyces* sp. reduced the TAN content of the silages, while *Neocallimastix* sp. increased it.

Table 73 The main effects of anaerobic fungal inoculants on the chemical composition (g / kg DM) of various forage silages after 28 days of inoculation.

	Forage silages				
Parameter	***A.gayanus***	***B.decumbens***	***L. perenne***	***T. aestivum***	**SEM**
Chemical constituent (g/kg DM)					
DM (g/kg)	971.53^{b}	971.53^{b}	970.79^{b}	977.26^{a}	0.616
Organic matter	962.79^{a}	961.27^{a}	955.88^{b}	960.46^{a}	0.946
Ash	37.21^{c}	38.73^{b}	44.12^{a}	39.54^{b}	0.946
Crude protein	115.60^{b}	59.63^{c}	157.52^{a}	32.19^{d}	0.729
Neutral Detergent Fibre	835.42^{c}	840.29^{b}	750.91^{d}	859.80^{a}	0.332
Acid detergent Fibre	575.07^{b}	566.81^{c}	530.80^{d}	588.23^{a}	0.861
Hemicellulose	260.34^{b}	273.47^{a}	220.11^{c}	271.57^{a}	0.807
Secondary metabolites (g/kg DM)					
Total phenols	10.11^{b}	8.47^{c}	11.24^{a}	7.15^{d}	0.247
Total tannins	5.54^{a}	4.24^{b}	5.12^{a}	3.70^{c}	0.133
Total antioxidant	4.64^{b}	4.00^{c}	6.45^{a}	2.71^{d}	0.170
	Fungal inoculants				
Parameters	**Control**	***Neocallimastix***	***Orpinomyces***	**SEM**	
Chemical constituents (g/kg DM)					
DM (g/kg)	977.45^{a}	971.59^{b}	969.29^{c}	0.533	
Organic matter	967.51^{a}	956.08^{b}	956.72^{b}	0.820	
Ash	32.50^{b}	43.92^{a}	43.28^{a}	0.820	
Crude protein	83.76^{b}	93.11^{a}	92.84^{a}	0.632	
Neutral Detergent Fibre	831.88^{a}	817.49^{b}	815.44^{c}	0.288	
Acid Detergent Fibre	571.47^{a}	562.80^{b}	561.42^{b}	0.746	
Hemicellulose	260.41^{a}	254.69^{b}	254.02^{b}	0.699	
Secondary metabolites (g / kg DM)					
Total phenols	9.89^{a}	8.68^{b}	9.16^{ab}	0.214	
Total tannins	4.70^{a}	4.50^{a}	4.76^{a}	0.115	
Total antioxidant	4.42^{a}	4.68^{a}	4.24^{a}	0.147	

Means with different letters in the same row are significantly ($P < 0.05$) different. SEM; standard error of means

The interactive effects of forage silage and fungal inoculants produced a significant ($P < 0.05$) effect on the chemical composition of the silages after 28 days of ensiling (Table 74). The fungal inoculants reduced the DM, OM, NDF, ADF, and hemicelluloses contents, but increased the CP and ash contents of the forage silages. The increase in the CP content of each of the British forage silages (i.e. TAS and LPS) was not different from their un-inoculated controls, but that of the tropical forage silages (i.e. BDS and AGS) was different from their un-inoculated controls. *Neocallimastix* sp. reduced fibre and hemicelluloses contents of AGS and LPS more than *Orpinomyces* sp., while the opposite was recorded in BDS. The fungal inoculants reduced fibre and hemicelluloses in a similar pattern in TAS. In all of the forage silages, the fungal inoculants also increased the CP content in a similar pattern.

The fungal inoculants reduced the TP content of all the forage silages. *Orpinomyces* sp. increased the TT content of almost all the forage silages except LPS, while *Neocallimastix* sp. reduced the TT content of almost all the forage silages except in AGS. The fungal inoculants reduced the TAN content of LPS and TAS, while they increased the TAN content of AGS and BDS, with *Neocallimastix* sp. recording a greater increase in TAN than *Orpinomyces* sp.

Table 74 The interactive effects of forage silage and anaerobic fungal inoculants on the chemical composition (g / kg DM) and secondary metabolites (g / kg DM) after 28 days of inoculation

		Chemical contents (g/ kg DM)				Fibre contents (g / kg DM)			Metabolites (g / kg DM)		
Forage silages	**Fungal inoculants**	**DM**	**OM**	**Ash**	**CP**	**NDF**	**ADF**	**HEM**	**TP**	**TT**	**TAN**
AG	Control	976.65^{ab}	968.89^{ab}	31.11^{f}	109.64^{c}	843.23^{d}	579.90^{bc}	263.33^{cd}	10.27^{b}	5.31^{abc}	3.34^{efg}
	Neocallimastix	970.83^{cde}	957.16^{cdef}	42.84^{abcd}	119.96^{b}	832.55^{fg}	574.16^{cd}	258.39^{d}	10.06^{b}	5.94^{a}	5.41^{bc}
	Orpinomyces	967.11^{e}	962.32^{abcd}	37.68^{cdef}	117.19^{b}	830.47^{g}	571.15^{d}	259.32^{d}	10.01^{b}	5.36^{ab}	5.15^{bcd}
BD	Control	977.88^{ab}	969.86^{a}	30.14^{f}	55.61^{e}	851.03^{c}	573.16^{cd}	277.87^{a}	9.06^{bc}	4.22^{cdef}	3.72^{ef}
	Neocallimastix	970.72^{cde}	954.92^{def}	45.08^{abc}	61.17^{de}	833.86^{ef}	566.64^{de}	267.22^{bc}	7.52^{c}	3.68^{ef}	4.40^{de}
	Orpinomyces	965.99^{e}	959.04^{bcde}	40.96^{bcde}	62.11^{d}	835.97^{e}	560.64^{e}	275.33^{a}	8.85^{bc}	4.82^{bcd}	3.87^{def}
LP	Control	974.49^{bcd}	966.73^{ab}	33.27^{ef}	154.78^{a}	768.99^{h}	540.88^{f}	228.11^{e}	13.08^{a}	5.61^{ab}	7.49^{a}
	Neocallimastix	969.19^{de}	951.72^{ef}	48.28^{ab}	158.21^{a}	745.44^{i}	523.67^{g}	221.78^{e}	10.06^{b}	4.76^{bcde}	6.03^{b}
	Orpinomyces	968.69^{e}	949.18^{f}	50.82^{a}	159.58^{a}	738.29^{j}	527.86^{g}	210.43^{f}	10.60^{b}	5.00^{abc}	5.84^{b}
TA	Control	980.80^{a}	964.54^{abc}	35.46^{def}	31.01^{f}	864.28^{a}	591.94^{a}	272.34^{ab}	7.17^{c}	3.64^{f}	3.13^{efg}
	Neocallimastix	975.61^{abc}	960.51^{bcd}	39.49^{cde}	33.10^{f}	858.10^{b}	586.74^{ab}	271.36^{ab}	7.10^{c}	3.61^{f}	2.87^{fg}
	Orpinomyces	975.38^{abc}	956.33^{cdef}	43.67^{abcd}	32.47^{f}	857.03^{b}	586.01^{ab}	271.02^{ab}	7.19^{c}	3.85^{def}	2.11^{g}
Pooled SEM		1.1	1.6	1.6	1.3	0.6	1.5	1.4	0.4	0.2	0.3

Means with different letters in the same column are significantly ($P < 0.05$) different. AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent fibre, ADF; acid detergent fibre, HEM; hemicellulose, TP; total phenols, TT; total tannins, TAN; total antioxidant, SEM; standard error of means.

Forage silages with anaerobic fungal inoculants recorded significant ($P < 0.05$) losses (%) in DM, OM, NDF, and consequently hemicelluloses loss, as well as an insignificant ($P < 0.05$) CP gain and significant ($P < 0.05$) ash gain after 28 days of inoculation (Table 75). An irregular pattern was recorded in the secondary metabolite contents, in that innoculation either led to a gross significant ($P < 0.05$) loss or gain in the TP, TT and TAN of the forages. In general, the fungal inoculants led to minimal nutrient losses (< 5%) in. DM, OM, NDF, ADF and Hem , higher ash gain, and minimal CP gain (< 10%) in each of the forage silages. *Orpinomyces* sp. recorded more fibre and hemicelluloses losses in AGS, LPS and TAS than *Neocallimastix* sp., while the opposite was recorded in BDS. The fungal inoculants produced identical CP gain in each of the ensiled forages. The fungal inoculants led to similar metabolite losses (%) in each of the ensiled forages. However, more TP loss (< 17%) was recorded in LPS and BDS than in AGS and TAS. *Neocallimastix* sp. led to TT losses in most of the forage silages except in AGS, while *Orpinomyces* sp. led to TT gain in most of the forage silages except in LPS. The fungal inoculants led to TAN loss in LPS and TAS, while they led to TAN gain in AGS and BDS. The highest TAN gain (< 50%) was recorded in AGS and the highest TAN loss (< 30%) was recorded in TAS.

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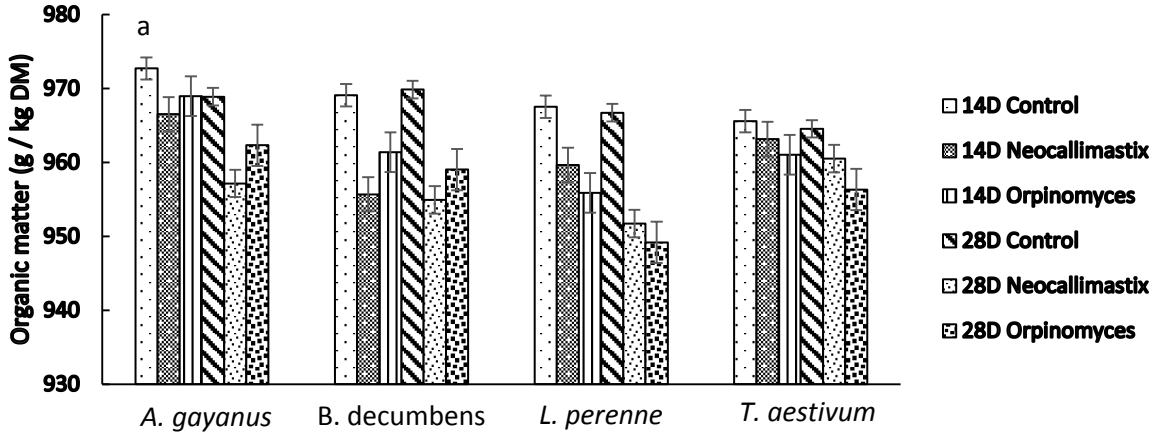
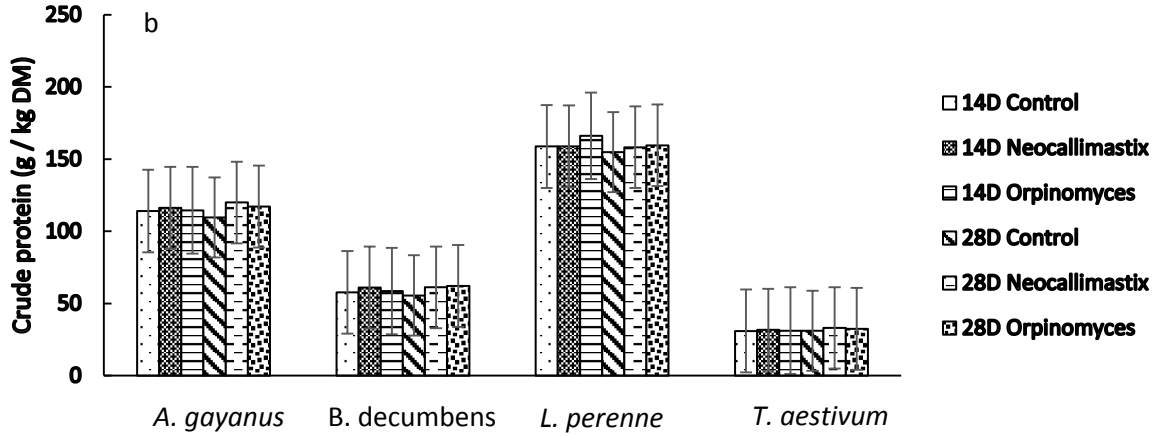
Table 75 The interactive effects of forage silage and anaerobic rumen fungal inoculants on nutrient loss (+) / gain (-) % of ensiled forages after 28 days of inoculation compared to control

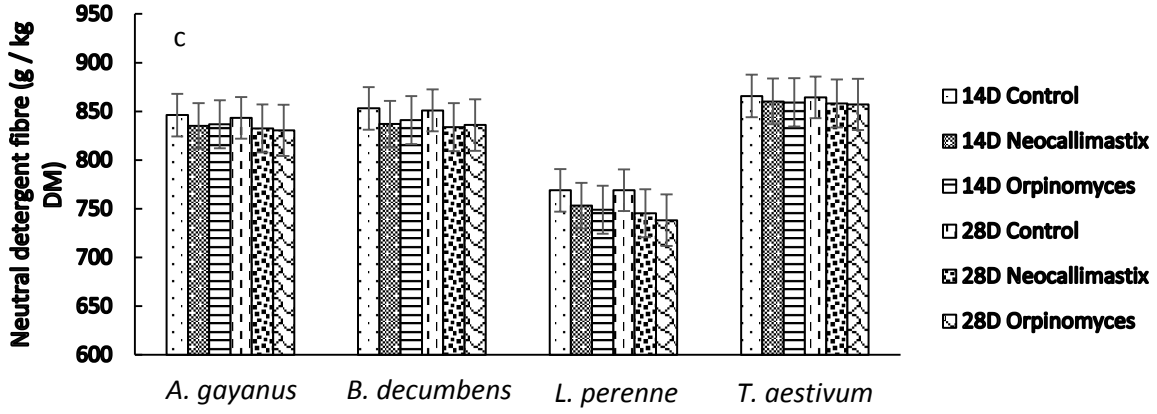
		Chemical contents loss/gain (%)				Fibre contents loss/gain (%)			Metabolites loss/gain (%)		
Forage	**Fungi**	**DM**	**OM**	**Ash**	**CP**	**NDF**	**ADF**	**HEM**	**TP**	**TT**	**TAN**
AG	*Neocallimastix*	0.60^{b}	1.21^{abc}	-37.71^{abc}	-9.41	1.27^{e}	0.99^{b}	1.87^{b}	2.01^{b}	-11.76^{b}	-61.79^{e}
	Orpinomyces	0.98^{ab}	0.68^{bc}	-21.12^{ab}	-6.88	1.51^{de}	1.51^{b}	1.52^{b}	2.48^{b}	$- 0.99^{ab}$	-54.09^{e}
BD	*Neocallimastix*	0.73^{ab}	1.54^{ab}	-49.56^{bc}	-10.0	2.02^{c}	1.14^{b}	3.83^{b}	17.01^{a}	12.75^{a}	-18.39^{d}
	Orpinomyces	1.22^{a}	1.12^{abc}	-35.90^{abc}	-11.7	1.77^{cd}	2.19^{ab}	0.92^{b}	2.30^{b}	-14.23^{b}	-4.10^{cd}
LP	*Neocallimastix*	0.54^{b}	1.55^{ab}	-45.12^{bc}	-2.21	3.06^{b}	3.18^{a}	2.78^{b}	23.10^{a}	15.14^{a}	19.53^{ab}
	Orpinomyces	0.60^{b}	1.82^{a}	-52.74^{c}	-3.1	3.99^{a}	2.41^{ab}	7.75^{a}	18.90^{a}	10.95^{a}	22.06^{ab}
TA	*Neocallimastix*	0.53^{b}	0.42^{c}	-11.35^{a}	-6.73	0.72^{f}	0.88^{b}	0.36^{b}	1.00^{b}	0.88^{ab}	8.28^{ab}
	Orpinomyces	0.55^{b}	0.85^{abc}	-23.14^{abc}	-4.69	0.84^{f}	1.00^{b}	0.49^{b}	-0.27^{b}	-5.60^{ab}	32.68^{a}
Pooled SEM		0.115	0.197	6.23	2.46	0.077	0.327	0.721	2.421	4.622	3.551

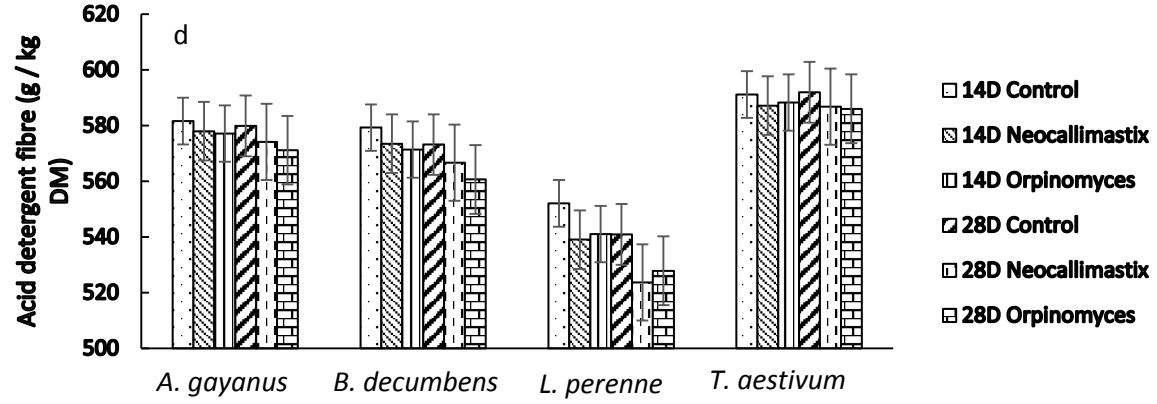
Means with different letters in the same column are significantly ($P < 0.05$) different. AG; *A. gayanus*, BD; *B. decumbens*, LP; *L. perenne*, TA; *T. aestivum*, DM; dry matter, OM; organic matter, CP; crude protein, NDF; neutral detergent fibre, ADF; acid detergent fibre, HEM; hemicellulose, TP; total phenols, TT; total tannins, TAN; total antioxidant, SEM; standard error of means.

The OM, CP, NDF, ADF, Hemicellulose, TP, TT and TA contents of the ensiled forages inoculated with anaerobic rumen fungi over two incubation times (i.e. 14 and 28 days) are shown in Fig 13: a to h respectively. The OM and fibre contents of the ensiled forages inoculated with anaerobic rumen fungi decreased with an increase in the incubation time. The CP content of the ensiled forages increased with an increase in the incubation time. The hemicellulose content of the ensiled forages obtained after 14 days of inoculation seems similar to those ensiled forages obtained after 28 days of inoculation. The TP and the TT contents of the treated forages increased with increase in the ensiling time, except in the reduced TP and TT contents recorded in *Neocallimastix* treated *L. perenne* after 28 days of inoculation. The TA contents of the ensiled forages were increased with *Neocallimastix* treatment, while they were decreased with *Orpinomyces* treatment after 28 days of inoculation respectively.


a
Organic matter (g / kg DM)
980
970
960
950
940
930
A. gayanus
B. decumbens
L. perenne
T. aestivum
14D Control
14D Neocallimastix
14D Orpinomyces
28D Control
28D Neocallimastix
28D Orpinomyces


b
Crude protein (g / kg DM)
250
200
150
100
50
0
A. gayanus
B. decumbens
L. perenne
T. aestivum
14D Control
14D Neocallimastix
14D Orpinomyces
28D Control
28D Neocallimastix
28D Orpinomyces


c
Neutral detergent fibre (g / kg DM)
950
900
850
800
750
700
650
600
A. gayanus
B. decumbens
L. perenne
T. aestivum
14D Control
14D Neocallimastix
14D Orpinomyces
28D Control
28D Neocallimastix
28D Orpinomyces


d
Acid detergent fibre (g / kg DM)
620
600
580
560
540
520
500
A. gayanus
B. decumbens
L. perenne
T. aestivum
14D Control
14D Neocallimastix
14D Orpinomyces
28D Control
28D Neocallimastix
28D Orpinomyces

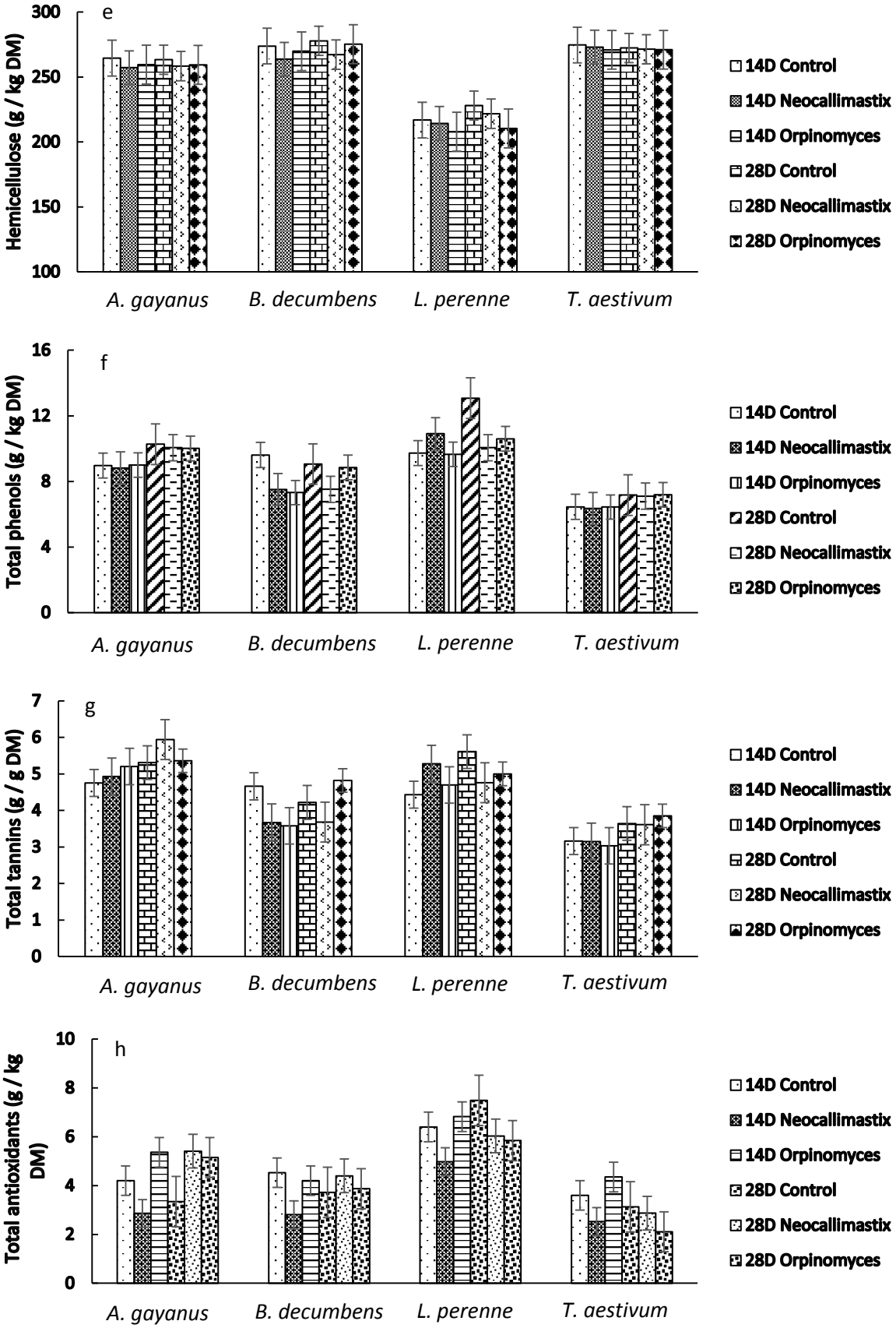


Figure 13 a-h show the chemical composition and secondary metabolites content of forages with 3 different fungal treatments (Control, Neocallimastix and Orpinomyces) respectively for 14 and 28 days.

7.4 Discussion

The study aimed to improve the quality of the selected forages by ensiling them with anaerobic rumen fungal inoculants, so as to facilitate the fermentation stage of the silage procedure. The fungal inoculants used were capable of improving the quality of the forages and improved the fermentation rate with lowered pH, increased CP content, increased fibre degradation, increased metabolite content, and increased total antioxidant content of the tropical forages, with minimal nutrient losses in the silages. Many researchers, such as McDonald *et al.* (1991); Weinberg and Muck (1996); Cai *et al.* (1999); Holzer *et al.* (2003); Xing *et al.* (2009); Tian *et al.* (2014); (Lee *et al.*, 2015); Khota *et al.* (2016); Pholsen *et al.* (2016); Khota *et al.* (2018), have reported that the use of microbial inoculants improved silage quality if the inoculants improved the fermentation characteristics (i.e. reduced pH, reduced protein degradation that led to lower ammonia nitrogen and butyric fermentation, increased lactic acid content, increased water-soluble content needed by microbes to produce lactic acid). The researchers also found an increase in specific metabolites that were required in inhibiting the growth and activity of spoilage microbes, alongside increased CP content, improved fibre degradation, and improved nutrient degradability or digestibility with minimal energy losses in the fungal inoculated silages.

During ensiling, pH reduction is expected. This is because, during fermentation of sugars or water-soluble carbohydrates, volatile fatty acids are released for microbial utilisation towards lactic acid production. It is the lactic acid that lowers the pH to aid in the silage stability, so as to minimise the growth of microbes involved in biological degradation and conserve higher amount of digestible nutrients, i.e. protein and energy, that will be needed in animal feed (McDonald *et al.*, 1991; Muck and Shinners, 2001; Lee *et al.*, 2015). The reduction in pH by anaerobic rumen fungal inoculants in this study indicates that the inoculants are facilitating the ensiling process. The outcome of this study supports the findings of Lee *et al.* (2015), where the use of anaerobic rumen fungi facilitated reduced pH in silage production.

Optimal pH for anaerobic fungi is reported by Orpin and Joblin (1997) to be between 6 and 7, above or below which can be detrimental to the fungal growth and activity. However, even at the 28th day of ensiling the forages with fungal inoculants, there was still evidence of fungal activity through increased CP content and a bit fibre reduction, but the fungal population was not measured in this study. This outcome was supported by the report of Lee *et al.* (2015), where the anaerobic fungal population in silage was increased up to 30 days, and increased activity was also recorded through increased CP content and fibre degradation. In this study the

pH reduction in the forages ensiled with fungal inoculants was not as rapid as those reported by Lee *et al.* (2015). Also, the forages ensiled in this study varied in their responses in pH reduction. The variations recorded could be attributable to the variation in the forage water-soluble carbohydrate contents, as the unavailability of soluble carbohydrates leads to insufficient sugars needed for lactic acid production that is required to reduce the pH (Khota *et al.*, 2016). Also, different forages produce different acid buffering capacity, i.e. forages with more sugars tend to reduce pH more rapidly because of their lower acid buffering capacity than forages without soluble sugars (Zhang *et al.*, 2016). The fungal inoculation improved the silage quality through decreased pH values in the ensiled forages; however, the pH was not reduced to less than 4, that is usually the pH when silage is stabilised (Khota *et al.*, 2016; Pholsen *et al.*, 2016). This may be attributable to the time used for ensiling, as well as the water soluble carbohydrate contents of the forages (Khota *et al.*, 2016; Pholsen *et al.*, 2016). Lower pH is required to stabilise the silage, but the growth and activity of the ARF can be limited, so there is the need to improve the ARF growth and activity within the first 28-30 days, especially their enzymatic activity for an increased fermentation rate.

The increased CP content and fibre degradation in the ensiled forages inoculated with ARF is evidence that the fungal inoculants are exhibiting their proteolytic and hydrolytic enzymatic function (Xing *et al.*, 2009; Lee *et al.*, 2015). This function is needed to increase the water-soluble carbohydrates required by microbes involved in the production of lactic acid (Cai *et al.*, 1999).

The increased CP content could be as a result of the solubility of structural carbohydrates that facilitated fungal growth in anticipation for the degradation of all dietary nitrogen content (i.e. both degradable and indegradable dietary nitrogen) for protein synthesis (Frumholtz *et al.*, 1989; Fondevila *et al.*, 1990; Russell *et al.*, 1992; Van Soest, 1994) or can be attributed to the contributing effect of fungal biomass (Denman and McSweeney, 2006). The insignificant CP increase obtained in some of the forage silages inoculated with ARF might be that the contribution of fungal biomass towards total CP content is not making a significant difference. The obtained insignificant increase in CP content of some of the inoculated ensiled forages was similar to that reported by Lee *et al.* (2015) when the effect of three anaerobic fungal inoculates on the fermentation characteristics of rice straw silages was investigated. Also, the increased CP content of the inoculated ensiled forages with an increase in inoculation time was in support of the report of Lee *et al.* (2015). This reflects that the fungi are growing and they are increasing in biomass weight (Fazaeli, 2007). The few cases where a significant increase in CP content

was recorded can be attributed to the C_4 (i.e. tropical plants in hot climate) plants. This supports the statement that C_4 plants generally do contain lower CP contents, but they are more efficiently utilised by microbes than C_3 (i.e. temperate plants in cold climate) plants (Wilson *et al.*, 1976). In general, the quality of the silages was increased by the fungal inoculation through increased CP content.

The increased fibre degradation in inoculated ensiled forages was in agreement with previous findings that most anaerobic rumen fungi were capable of degrading fibre, due to their enzymatic activities and the ability of their mycelium/rhizoids or bulbous hold fast to penetrate structural carbohydrates (Gordon and Phillips, 1998; Abrão *et al.*, 2014; Puniya *et al.*, 2014). It is also an indication that the fungi are exhibiting their fibrolytic activity (i.e. cellulases and xylanases) in decomposing the structural carbohydrates (Gordon and Phillips, 1998). Hydrolytic enzymes like cellulases have been used by researchers in improving the silage quality in that it aids in fibre degradation, thus making water-soluble carbohydrates available for use by lactic acid bacteria. The bacteria, therefore, produce lactic acid needed for pH reduction (Eun *et al.*, 2006; Eun and Beauchemin, 2008; Xing *et al.*, 2009; Khota *et al.*, 2016). In general, the fungal inoculants varied in their preferences for the substrate in which they exhibit higher fibre degradation. *Orpinomyces* sp had greater preference for LPS and TAS, while *Neocallimastix* sp had greater preference for AGS and BDS. This can be attributed to fungal strains and forage chemical composition, as different fungal strains vary in their fibrolytic roles (Ho and Abdullah, 1999; Paul *et al.*, 2004), while forage chemical composition influences their ability to support the growth (Puniya *et al.*, 2014) and activity (Gordon and Phillips, 1989) of fungi. The reduced fibre content of LPS and TAS by *Orpinomyces* sp. inoculant was in agreement with the findings of Lee *et al.* (2015), where *Orpinomyces* recorded higher reduction in fibre content of rice straw silages than *Neocallimastix* and *Piromyces* anaerobic fungal strains. The reduced fibre contents in all the forage silages with increased inoculation time is an indication that the fibrolytic enzymatic activity of the selected anaerobic rumen fungi was still active up till the 28th day. This is in support of the findings of Yue *et al.* (2009b), where some of the fibrolytic enzymes function of *Neocallimastix* investigated increased with increase in incubation time. The high fibre content of the forage silages, despite fungal inoculation which has reduced it to a certain extent, might be attributed to the maturity stage of the forages, as highly matured forages contain more structural carbohydrates that can negatively influence the achievement of good quality silage (Silva *et al.*, 2018). This supports the report of Pholsen *et al.* (2016) that high-quality silages using tropical forages are difficult

to produce, due to their low water-soluble carbohydrates and presence of natural lactic acid bacteria. Although, the fungal inoculation improved the forages silage quality through fibre degradation, and the sites in which the degradation takes place would have been exposed for accessibility by other microbes, there is the need to investigate these silages in an environment such as the rumen, where many microbes exist for an efficient degradation of the silages thus increasing nutrient utilisation.

The continued increase in metabolites (i.e. TT and TP) content up to the 28th day in almost all forage silages inoculated with *Orpinomyces* sp. , as well as the increased TAN content up to the 28th day in tropical forages, were in agreement with the findings that the use of additives in controlling the microbial silage processes leads to the production of specific metabolites that are involved in the inhibition of the growth and activity of spoilage microbes (e.g. *Clostridium* sp.) in the silage (Weinberg and Muck, 1996; Holzer *et al.*, 2003; Neureiter *et al.*, 2005). The continued reduction in metabolite (i.e. TT and TP) contents recorded in almost all forage silages inoculated with *Neocallimastix* sp. was not expected. However, the increased TAN content up to the 28th day in tropical forage silages might be because the fungus was producing TAN as its specific metabolite. The increased TAN has other benefits, in that it is involved in maintaining a balance in the animal health and immune system (Chew, 1995). The variation in time at which each fungal inoculant was supporting metabolites and TAN, can be attributable to the fact that fungi vary in the rate and fermentation time in which they achieve lignocellulose degradation (Bauchop, 1981; Orpin, 1981; Gordon and Phillips, 1989; Hoover and Stokes, 1991; Ho and Abdullah, 1999), from which phenolic compounds are being released. *Orpinomyces* was capable of releasing TAN at an earlier inoculation time, while *Neocallimastix* was capable of achieving it at a later inoculation time. The increased metabolite and TAN contents recorded in the inoculated ensiled forage silages as the inoculation time increased might be because the fungal inoculants are either secreting some metabolites, as reported by Paul *et al.* (2004), or that their extensive lignocellulose degradation led to the dissociation of lignified tissues from which more phenolic compounds were released (Akin *et al.*, 1983; Akin and Borneman, 1990; Ho and Abdullah, 1999). The fungal inoculants, especially *Orpinomyces* sp., increased the TT and TP contents up to the 28th day in almost all the forage silages, as well as increasing TAN contents of the tropical forage silages up to the 28th day, while *Neocallimastix* sp. increased the TAN content up to the 28th day in almost all the forage silages and reduced metabolite contents in the forage silages.

The minimal energy loss (i.e. < 5%) and CP gain in the inoculated forage silages are an indication that the nutrients are well preserved and not utilised by spoilage microbes, which is an attribute of good quality silage (Neureiter *et al.*, 2005). In addition, the goal of ensiling was also achieved, which was to minimise nutrient degradation by spoilage microbes and conserve a significant amount of digestible energy and protein for the ruminant animals (Shaver, 1992; Muck and Kung Jr, 1997). However, the higher metabolites loss (> 40%) with the use of *Neocallimastix* sp., which was complemented with higher TAN gain, was not expected. It might be that the fungus was utilising the metabolites towards increasing the TAN capacity of forages, therefore using the TAN as its specific metabolite that is needed for the inhibition of spoilage microbes (Neureiter *et al.*, 2005). The use of the fungal inoculant was capable of improving the silage quality through minimised energy losses and CP gain. *Orpinomyces* sp. inoculation of the forages silage led to metabolites gain and TAN gain of the tropical forage silages. *Neocallimastix* sp. led to higher TAN gain and metabolite loss in the tropical forage silages.

7.5 Conclusion

The supplementation of the isolated anaerobic rumen fungi as silage inoculants improved the silage quality of the forages. The improvement was reflected in the reduced pH, increased CP content, decreased fibre content, increased metabolite contents especially by *Orpninomyces* sp., and increased TAN contents especially by *Neocallimastix* sp. This improvement was complemented with a minimal nutrient loss. *Orpinomyces* sp. had a greater preference in degrading fibre in the temperate forage silages, i.e. LPS and TAS, while *Neocallimastix* sp. had a greater preference in degrading fibre in the tropical forage silages, i.e. AGS and BDS. Although the silage was improved, the fibre content was still high but there is the possibility that the chemical bonds that exists between lignin and other cell wall contents, i.e. cellulose and hemicellulose, might have been loosened during ensiling. These ensiled forages therefore require further degradation by rumen microbes for proper utilisation of nutrients by ruminant animals.

7.6 Recommendation

Further research is, therefore, necessary to obtain an efficient nutrient utilisation by animals consuming these silages by introducing the forage silages into the rumen environment, with or without the isolated ARF to manipulate the rumen microbial ecosystem for an increase in the fermentation rate.

In addition, more research is required to improve the fermentation rate of the ARF when used as inoculants in silage procedures over a short period (28-30 days). This can be achieved by using natural lactic acid bacteria (e.g. *Lactobacillus plantarium* and *L. casei*), that exist naturally on tropical grasses and that can survive under low pH, along with the ARF as a source of enzymatic function. Further tests should be carried out to determine the rate and extent of rumen degradation (*in vitro* and *in sacco*), digestion (*in vivo*) and palatability of similar fungal treated silages before their farm scale testing for utilisation by ruminant animals.

Chapter 8

General Summary, discussion and recommendations

8.1 General Summary

In tropical countries such as Nigeria, the scarcity and unavailability of good quality green fodders during the dry season has been the major problem facing the livestock industry, especially ruminant animals. The available feeds are not capable of meeting the nutritional needs of ruminant animals, thus reducing the animals' production and performance at this period. Therefore animal nutritionists look for ways to nutritionally improve these feeds. In the quest to find ways of improving the feeds, researchers are trying hard to identify improvement methods that are not hazardous to the animal, people and environment; less time consuming; less capital intensive; and capable of producing an end product with improved total antioxidant properties and anti-methanogenic properties.

The use of biological methods for feed improvement has been identified to be capable of fulfilling these purposes. This can be achieved with the use of several biological agents (i.e. bacteria, yeast, fungi etc.), among which fungi are greatly preferred due to their high enzymatic activity (Ho and Abdullah, 1999; Mahesh and Mohini, 2013) and possession of rhizoids or hyphae (Gordon and Phillips, 1998; Abdel-Hamid *et al.*, 2013; Mahesh and Mohini, 2013; Abrão *et al.*, 2014; Leis *et al.*, 2014) that penetrate complex intra-cellular plant tissues that act as barriers, thus degrading lignocelluloses in the feeds. The fungi can either be aerobic or anaerobic, and they vary in the way they improve the utilization of low-quality feed for better ruminant performance.

The aerobic fungi improve the potential use of these feeds by degrading lignin, which is the major cell wall component of plants that physically restricts the bioavailability of other cell wall components (Pérez *et al.*, 2002; van Kuijk *et al.*, 2015; Daniel, 2016) used in the rumen. On the other hand, the anaerobic fungi are used as additives with these low quality feeds, either as direct fed or as silage inoculants, in order to manipulate the rumen microbial ecosystem by increasing the number and activity of cellulolytic microbes in the rumen (Dayananda *et al.*, 2007; Tripathi *et al.*, 2007b; Tripathi *et al.*, 2007a) and thus altering the fermentation pathway which leads to an increased nutrient utilization (Thareja *et al.*, 2006; Sehgal *et al.*, 2008). However, there is still a need to investigate their role in improving low-quality forages as their functions seem to vary.

Therefore, a series of studies were carried out to evaluate the effectiveness of both aerobic and anaerobic fungi in improving the nutrient utilization of some of the most available and abundant forages in Nigeria, alongside two commonly used British forages. To achieve this, six Nigerian forages, which included four grasses (*Pennisetum purpureum, Panicum maximum, Brachairia decumbens* and *Andropogon gayanus*) and two fodder tree legumes (*Gliricidia sepium* and *Leucaena leucocephala)* as well as two British forages which included one grass (*Lolium perenne*) and one straw (*Triticum aestivum*), were firstly investigated for their nutritive values and secondly their influence on rumen microbes. This study was carried out to ascertain if they are appropriate forages that would possibly benefit from improvement in nutrient content, rumen degradation and total digestive tract digestibility. The British forages were included in this series to serve as positive (*L. perenne*) and negative (*T. aestivum*) controls for the Nigerian forages, thus helping to place the results in perspective.

Two grasses (i.e. *B. decumbens* and *A. gayanus*) and one tree legume (i.e. *L. leucocephala*) were eventually selected for the main study. The grasses were selected based on the fact that they showed the lowest nutritive quality, lowest nutrient utilisation by rumen microbes, and the highest methane gas production. They also supported higher bacterial or fungal counts, which is an indication that they can be biologically improved as they seem to encourage more microbial multiplication. The legume was selected based on its ability to be used as a protein supplement, increased ammonia content and TAC, reduced methane production, reduced short-chain fatty acids and increased microbial growth. This indicates that it can be used as a protein supplement and not as an energy source, anti-methanogenic agent or used to support microbial multiplication in the rumen. Originally, the selected legume was to be used as a supplement with the pre-treated forages in either *in sacco* or *in vivo* trials, but based on time and finance, this experiment was not carried out. This made the two selected Nigerian grasses and the British forages to be the main focus in the aerobic and anaerobic fungal studies.

Regarding the aerobic studies, two white rot aerobic fungi (*Pleurotus ostreatus*; PO and *Ceriporiopsis rivulosus*; CR) were selected, based on their ability to improve low-quality substrates as well as on their frequency of use in literature. *P. ostreatus* has been extensively researched, while *C. rivulosus* has not been widely studied. In preparation for their use for the upgrade, they were firstly investigated for the best growth conditions in the absence of any fibrous substrate. The use of Potato dextrose agar at 20^0C supported the growth and activity of *C. rivulosus,* while the use of Malt extract agar at 30^0C supported the growth and activity of *P. ostreatus.* Under each of these ascertained growth conditions for each fungus, agar plugs were

obtained and used for the substrate inoculation over 14 and 28 days respectively. The objective of the fungal pre-treatment of the forages was to improve their nutritive value. However, there are several reports where fungal treatment does not improve the nutritive value of forages. This research, therefore, investigated one possible reason that may have caused this, which is the removal of solubles caused by improper handling of pre-treated forages. This research investigated the extent of loss of solubles using either a pump (PFF) or free flow filtering (FFF) method. To quantify the release of solubles in the pre-treated forages, the effects of two factors, i.e. substrate-liquid ratio and incubation time, were evaluated.

In this study, both the PFF and FFF method resulted in soluble losses in the fungal pre-treated forages. Losses with the PFF method were higher and more consistent in terms of volume than the losses obtained from the FFF method, due to the pump assisted filtration. Therefore, the filtered residues from the PFF method were studied further for their *in vitro* degradability over time. The PFF method did negatively influence the ability of the fungi to improve the chemical composition of the pre-treated forages in comparison with the FFF method. Also, the PFF method did not improve the *in vitro* nutrient degradability and *in vitro* fermentation parameters of the fungal-pre-treated forages. On the other hand, based on the nutrient composition of the pretreated forages obtained from the FFF method which contained more digestible fraction, the fungal pre-treated forages are predicted to have a higher digestibility than their untreated counterparts. A substrate-liquid ratio of 1:5 supported increased activity and growth of *C. rivulosus* on all the forages, and of *P. ostreatus* on some forages except *A. gayanus* and *B. decumbens*. Short (14 day) incubation times supported both fungal growth and activity on *L. perenne* and *T. aestivum*, but not on *A. gayanus* and *B. decumbens*, resulting in increased release of solubles, which are the main focus of this study.

In the anaerobic fungal studies, the improvement was evaluated by using selected fungi as inoculants for the selected low-quality forages in silos. In preparation for this possible improvement, fourteen anaerobic fungi were firstly isolated, screened and identified in our laboratory. The two fungi with the best growth performance were selected, which belonged to the genus *Neocallimastix* and *Opinomyces*. These fungi were used as inoculants over 2 inoculation times (i.e. 14 and 28 days) for the low-quality forages to improve their nutritive value and nutrient utilisation. The supplementation of these two anaerobic rumen fungi as silage inoculants improved the silage quality of the forages. This improvement was reflected in the reduced pH, increased CP content, decreased fibre content, increased metabolite contents especially by *Orpninomyces* sp., and increased TAN contents especially by *Neocallimastix* sp.

This improvement was complemented with a minimal nutrient loss. *Orpinomyces* sp. had a greater preference in degrading fibre in the temperate forage silages, i.e. *L. perenne* silage and *T. aestivum* silage, while *Neocallimastix* sp. had a greater preference in degrading fibre in the tropical forage silages, i.e. *A. gayanus* silage and *B. decumbens* silage. Although the silage was improved, the fibre content was still high, but there is the possibility that the chemical bonds that exist between lignin and other polysaccharides might have been loosened during ensiling. These ensiled forages, therefore, require further degradation by rumen microbes for the utilisation of nutrients by ruminant animals.

8.2 General Discussion

The aim of this study was to investigate the ability of i) aerobic fungi and ii) anaerobic fungi to improve the nutritive value and degradability of available dry season, low-quality feeds in such a way that the method is suitable for use by farmers, not hazardous to the animal, people and environment, less time consuming, less capital intensive, and capable of producing an end product with improved total antioxidant properties, anti-toxic properties and anti-methanogenic properties when utilised by the animals.

The aerobic fungi were unable to improve the nutritive value, especially nutrient degradability, of the pre-treated forages based on the processing method adopted. This may be because the PFF method led to the loss of solubles, and thus might have caused little or no change *in vitro* DMD. Nutritional composition improvement was achieved in the pre-treated forages, obtained with the FFF method, but not with the PFF method. Thus, it is possible that different results would have been obtained if the residues from the FFF method were studied for *in vitro* degradability. However, due to the collection of inconsistent volumes of liquids reflecting inconsistent loss of nutrients, these residues were not studied for *in vitro* DMD. The results provide an indication that the method adopted during processing of pre-treated forages is vital to the kind of improvement that would be recorded. Farmers should endeavour to ensure that losses of solubles are minimal and consistent by using containers/bags/sacks that will not allow the escape of solubles during pre-treatment, and also ensure that no pressure is exerted on the pre-treated forages. Nevertheless, none of the filtration methods (PFF or FFF) are recommended for their farm scale application during fungal improvement of forages.

The use of growth conditions favouring excessive growth and ligninolytic function by the fungi on the substrate should be avoided when implementing the upgrading of forages on the farm, as this leads to the release of more solubles that might be subsequently utilised by the fungi or

lost through improper handling. In this study the use of smaller particle size, *P. ostreatus*, the substrate-liquid ratio of 1:5 and the use of 28 days inoculation time led to more nutrient loss compared to when the substrate-liquid ratio of 1:3, *C. rivulosus*, and the use of 14 days inoculation time were used. This is because the latter conditions minimised the growth as well as the rate and extent of ligninolytic function, which makes these conditions more preferable for feedstock improvement. The use of growth conditions that supported higher laccase activity and a long inoculation time led to higher upgrade, but resulted in a higher nutrient loss in the upgraded forages. This dichotomy is a major drawback of the use of fungal pre-treatment, as the optimization of both is required for this method to effectively compete with the well-known physical, chemical and physico-chemical pre-treatment methods (van Kuijk *et al.*, 2016b). The use of purchased fungi should be avoided if possible, as it seems not to be economical, but fungi which are isolated or obtained from more affordable sources, such as a government agency having a cooperation with farmers in distributing these at low cost, might be more secure and/or be a better choice when working on the farm-based research.

In obtaining inocula for pre-treatment of a large amount of substrate, as is the case in the field, the use of agar plugs is not advisable due to increase in cost, time, complexity; these can't be produced on a large scale and might not be easy to use by farmers, and therefore may not be economical. Thus, the use of mushroom spawn produced from grains (i.e. wheat or millet) will be the preferred methodology, as this can be produced on a large scale, is simple, economical and easy to mix etc (Tripathi and Yadav, 1992; Sánchez, 2004). Although the adoption and the suitability of the process under field environments have not yet been adequately assessed, this seems to be the way forward. Clearly, there is still a great need to investigate the way these fungi act on the forages under field conditions.

The aerobic fungi did not improve the antioxidant properties of the pre-treated forages but generally led to a reduction in metabolites. This indicates that fungal treatment might be used as an option in improving potential dry season ruminant feeds such as fodder tree legumes in Nigeria that contain high levels of tannins (Cudjoe and Mlambo, 2014; Franzel *et al.*, 2014).

Treatment with aerobic fungi (using pre-treated forages obtained from the PFF method) reduced the methane gas production, but this was due to a reduction in degradability. There is the distinct possibility that increased methane gas production will be recorded with increased nutrient degradability if pre-treated forages from the FFF method were investigated for *in vitro* degradation and fermentation parameters. This is because the end products of fungal

degradation in the rumen do lead to the production of hydrogen (Ho and Abdullah, 1999), which is an essential requirement for the methanogens to synthesis methane in the rumen (Morgavi *et al.*, 2010). Therefore, if pre-treated forages are to be fed in the field with live animals, the inclusion of natural fodder tree legumes that contain metabolites with anti-methanogenic properties, such as *L. leucocephala* (Islam *et al.*, 1995; Franzel *et al.*, 2014) investigated in this study might be used as a supplementary feed.

The anaerobic rumen fungi (ARF) were also capable of improving the nutritive composition of low quality silages by degrading fibre components, increasing the soluble fraction, and improving fermentation through reduction in pH with minimal nutrient loss compared to aerobic fungi. The improvement in terms of lignocellulose degradation was minor; to obtain more improvement, the fermentation rate of the ARF needs to improve, possibly through the addition of natural lactic acid bacteria (e.g. *Lactobacillus plantarium* and *L. casei*) to the silages, as these can survive under low pH (Holzer *et al.*, 2003) along with the ARF which will serve as a source of enzymatic function. To further improve the nutrient utilisation when offered to animals, there is the need to offer such silages in the wet form, not dry, when utilised in the field. The surviving anaerobic fungi might then be swallowed along with the feed, as it has been researched that some ARF can survive exposure to air for some hours, ranging from 9 -13 hours (Orpin, 1981; Milne *et al.*, 1989; Struchtemeyer *et al.*, 2014). This may be further supplemented with additional live fungi (in liquid form) to aid further breakdown of lignocellulose in the rumen (Lee *et al.*, 2000a; Paul *et al.*, 2004; Nagpal *et al.*, 2011) which is their natural habitat.

The anaerobic fungi were capable of increasing the antioxidant properties of the forages, especially the Nigerian forages, more than the aerobic fungi, with *Orpinomyces* sp increasing the antioxidant content at an earlier period than *Neocallimastix* sp. This indicates that the use of anaerobic fungi in silages might improve the health and immune system of animals feeding on them, which is one of the benefits of anaerobic fungi as against aerobic fungi indicated in this study when grasses are offered. A longer period of inoculation with *Orpinomyces* led to a higher increase in secondary metabolites in comparison with with *Neocallimastix,* making *Orpinomyces* sp. more preferred for further silage studies, even though the organism required a relatively longer incubation time (i.e. 28-30 days). The use of *Neocallimastix* sp. resulted in better fibre degradation in tropical forages. while *Orpinomyces* sp. was the better in fibre degradation of the British forages. This implies that if the fungi are to be used as live cultures along with the silages, the selection of a more suitable probiotic may be beneficial on the basis of a forage type and amount. Although the suitability of the process on the farm has not yet

been investigated, this seems to be a way forward. The use of freshly isolated anaerobic fungi was time consuming and requires specific skills, and may therefore not be practical for individual farmers, but if isolated and sold cheaply to farmers in the form of paste or gel through a government agency or research institute, it may be a better choice.

8.3 Recommendations

Based on the various studies carried out so far, a few recommendations for future studies are listed as follows:

i. To determine the *in vitro* nutrient degradability, fermentation parameters and total gas production of the treated forages obtained from the free flow filtering (FFF) method to ascertain if the lesser loss of water-soluble contents does help improve the nutrient degradability and fermentation.

ii. To investigate the silages obtained from the anaerobic fungal inoculation for rumen degradability and fermentation parameters when fed along with living cultures of other anaerobic fungi, yeast or other suitable probiotics.

iii. To investigate the nutritive improvement that will be recorded when the most preferred anaerobic rumen fungi are used as inoculants for each silage (e.g. *Orpinomyces* sp. to the tropical forages and vice versa) along with lactic acid bacteria.

iv. To investigate the influence that feeding of the pre-treated forages (aerobic experiment) and silages fed along with live cultures (anaerobic experiment), with or without legume supplementation, will have on animal growth, nutrient digestibility and rumen fermentation..

v. To investigate the use of growth conditions (i.e. substrate: liquid ratio of 1:3, less inoculation time, i.e. 14 days, use of *C. rivulosus*, and larger particle size) that support less fungal growth and activity on the ability of the fungi to improve the nutritive value of treated forages in the field.

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APPENDICES

2.1 Dry matter (DM, AOAC official method 934.01)

Apparatus:

1. Alumunium foils and porcelain crucibles
2. Oven drier
3. Sample mill (Tecator Cyclotec 1093, Sweden)
4. A desiccator
5. Analytic weighing scale (Salter N&D, Japan)

Procedure:

Samples were oven dried at 60^0C for 48h. For fresh and wet STL, it was initially dried at 40^0C overnight before increasing the temperature to 60^0C to avoid any nutrient damage during drying. Dried samples then were ground to pass 1 mm sieve in a sample mill. DM was determined by oven drying representative samples in triplicate (0.5 g each in porcelain crucible) at 100^0C for 24 h. A desiccator was used to cool samples after being taken off the oven drier before weighing.

Equation:

C : Wt. of crucible (g)

CS_0 : Wt. of crucible with fresh sample (g)

S_0 : Wt. of fresh sample (g), $\mathbf{S_0 = CS_0 - C}$

CS_1 : Wt. of crucible with dried sample (g)

S_1 : Wt. of dried sample (g), $\mathbf{S_1 = CS_1 - C}$

$$\text{DM (g DM/ kg fresh sample)} = \frac{S_1}{S_0} \times 1000$$

2.1 Ash and Organic matter (AOAC official method 942.05)

Apparatus:

1. Furnace (Carbolite, AAF11/18, England)
2. Analytic weighing scale (Salter N&D, Japan)
3. Desiccator

Procedure:

The samples from DM analysis were then placed and ignited in a furnace at slowly rise temperature to 550^0C for 5 h. There were then removed and cooled in a desiccator before weighing them. Both ash and OM were expressed in g/kg DM.

Equation:

C : Wt. of crucible (g)

CS : Wt. of crucible with dried sample (g)

S : Wt. of dried sample (g), **S = CS-C**

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g), **A = CA-C**

$$\text{Ash (g/kg DM)} = \frac{A}{S} \times 1000$$

$$\text{OM (g/kg DM)} = \frac{S - A}{S} \times 1000$$

2.1 Ether extraxct (EE, AOAC official Method 920.39)

Apparatus:

1. A set of soxhlet extractor (thimble, flask , soxhlet extractor, heating mantle and condenser)
2. Analytic weighing scale (Salter N&D, Japan)
3. Cotton wool

Reagent:

(a) Solvent (petroleum ether 40-60^{o}C)

Procedure:

Adequate petroleum ether was placed into a pre-oven dried flask (overnight at 60^{o}C). On the other hand, about 1.5 g dried ground sample was placed into thimble and plug the top with cotton wool. It was then placed into the extractor and fitted to the flask. The next step was to fit the extractor and flask into the heating mantle and condenser. The flask was heated until the solvent gently boiled and allowing this extraction process for 6 h before removing. Finally, the residual solvent containing oils (EE) was oven dried for over the night at 60^{o}C and stored in a desiccator to cool before weighing.

Equation:

T : Wt. of thimble (g)

F : Wt. of flask (g)

TS : Wt. of thimble with dried sample (g)

S : Wt. of dried sample (g), **S = TS - T**

FE : Wt. of flask with ether extract

E : Wt. of ether extract (g), **E = FE - F**

$$EE\ (g/kg\ DM) = \frac{E}{S} \times 1000$$

2.1 Crude Protein (CP), Carbon and sulphur

Apparatus:

1. Elementar Vario Macro Cube (Germany). This machine can determine Nitrogen, carbon and sulphur in three-in-one process for a similar sample.
2. Analytic weighing scale (Salter N&D, Japan)
3. Thin foil cup

Procedure:

About 0.1 g of each dried ground sample was placed into a pre-tarred tin foil cup. It was then carefully folded and squashed into a pellet to expel the air and this was done by using a tool provided by Elementar. In particular to carbon (C) and nitrogen (N) determinations, the analysis was carried out in CN mode; this involved using a combustion, post combustion and reduction tube in the furnace of the analyzer. The combustion tube was at 930^{o}C and a sample was dropped into this via a carousel and ball valve. Oxygen was used to burn the sample and the gas was carried off in helium through both the post combustion and reduction tubes, which were also heated, to the detectors housed within the analyzer. Regarding to sulphur analysis, the combustion and reduction tubes were at 1150 ^{o}C and 850 ^{o}C respectively. Before each run a set of standards was run which ensured that the analyzer was working correctly. Standards were also run halfway through a sample run as well. To check that the analyzer has performed correctly there was a Daily Factor figure which was worked out after each run and this should lie between 0.9 and 1.1. Runs that did not meet these criteria were discarded. Each element was analyzed separately and a % figure was then obtained. CP content was calculated by multiplying N content with 6.25 and expresses CP in g/kg DM.

Equation:

F_c : Wt. of foil cup (g)

F_cS : Wt. of foil cup with sample (g)

S : Wt. of sample (g)

N_p : N content in percent (%)

N : N in gram (g), $\mathbf{N = N_p/100 \times 1000}$

CP (g/kg DM) = 6.25 X N

2.2 Neutral detergent fibre (NDF) (Van Soest *et al.*, 1991), neutral detergent fibre insoluble protein (NDFIP), neutral detergent fibre insoluble carbon (NDFIC)

Apparatus:

1. 100 ml tubes fit to the racks on digestion chamber
2. A set of digestion chamber (Gerhardt Kjeldaterm, Germany)
3. Sintered glass crucibles (porosity no. 1). They were initially washed and ashed at 550^{0} C for 3 hours, cooled in a desiccator, weighed and put back in a desiccator until ready to be used
4. A set of Buchner flask and vacuum pump

5. Glass rod stirrer
6. Elementar Vario Macro Cube (Germany), to analyze Nitogen and Carbon for NDFIP and NDFIC analyses.
7. pH metre

Reagents:

(a) Neutral detergent solution (ND); Adding 30 g Sodium dodecyl Sulphate, 18.61 g Di sodium dihydrogen EDTA, 6.81 g Di sodium tetraborate, 4.56 g Disodium hydrogen orthophoshphate, 10.0 ml tryethilene glycol into 1 L of distilled water and the pH of the solution was checked to be in the range of 6.9 - 7.1

(b) Acetone.

Procedure:

0.5 g each of dried ground sample was placed into the tubes. Then, 50 ml ND was added into it. After this, the tubes were placed on a set of digestion chamber. The temperature was set at 120^{o}C and it was reduced if rapid foaming happened to avoid splashing out. This extraction was lasted for 1 hour from a starting boiling. Next, tubes were taken out and each of them was swirled. The solution was then filtrated into a pre-weighed sintered glass crucible and completed the filtration using light vacuum suction. After this, the fibre residue on crucible was washed by filling two third of the crucible with hot (90-100^{0}C) water, stirred, soaked for few minutes and drained with the aid of vacuum suction. The sides of crucible were also rinsed. This washing was performed twice. It was then continued by having the same washing twice with acetone. The stirring rod was also rinsed before removing. Crucible with its content of fibre residual was oven dried at 100^{0}C overnight, cooled in a desiccator and weighed. About 0.1 g of dried fibre residue was taken for N and C analysis using Elementar Vario Macro Cube analyzer as described previously in order to get NDFIP and NDFIC. Finally, the remaining residual fibre content was ashed at 550°C in furnace for 5 h, cooled in a desiccators and weighed.

Equation:

F : Wt. of tube (g)

FS : Wt. of tube with dried sample (g)

S : Wt. of dried sample (g); **S = FS - F**

C : Wt. of sintered glass crucible (g)

CR : Wt. of sintered glass crucible with dried fibre residue (g)

R : Wt. of dried fibre residue (g); **R = CR - C**

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g) (after being corrected with the amount of fibre residue taken for N and C analysis)

N_p : N content in percent (%)

N : N in gram (g), **$N = N_p/100 \times 1000$**

C_p : Carbon in percent (%), **$C = C_p \times 1000$**

$$\text{NDF (g/kg DM)} = \frac{R - A}{S} \times 1000$$

$$\text{NDFIP (g/kg DM Fibre)} = \frac{(6.25 \times \mathbf{N})}{S} \times \text{NDF}$$

$$\text{NDFIC (g/kg DM Fibre)} = \frac{\mathbf{C}}{S} \times \text{NDF}$$

2.2. Acid detergent fibre (ADF) (Van Soest, 1990), acid detergent fibre insoluble protein (NDFIP), acid detergent fibre insoluble carbon (NDFIC)

Apparatus:

1. 100 ml tubes fit to the rack on digestion chamber
2. A set of digestion chamber (Gerhardt Kjeldaterm, Germany)
3. Sintered glass crucibles (porosity no. 1). They were initially washed, ashed at 550^0 C for 3 hours, cooled in desiccators, pre-weighed and put them back into the desiccator until ready to be used
4. A set of Buchner flask and vacuum pump
5. Glass rod stirer
6. Elementar Vario Macro Cube (Germany), to analyze Nitogen and Carbon for ADFIP and ADFIC analyses.

Reagents:

(a) Acid detergent solution (AD); Add 20 g cetyl trimethylammonium bromide (CTAB, technical grade) to 1 L 0.5M H_2SO_4 (added 27.7 ml H_2SO_4 (95-98%) to 972.3 ml H_2O)

(b) Acetone.

Procedure:

0.5 g each of dried ground sample was placed into the tubes. Then, 50 ml ND was added into it. After this, the tubes were placed on a set of digestion chamber. The temperature was set at 120ºC and it was reduced if rapid foaming happened to avoid splashing out. This extraction was lasted for 1 hour from a starting boiling. Next, tubes were taken out and each of them was swirled. The solution was then filtrated into a pre-weighed sintered glass crucible and complete filtration using light vacuum suction. After this, the fibre residue on crucible was washed by filling two third of the crucible with hot (90-100^{0}C) water, stirred, soaked for a few minutes and drained with the aid of vacuum suction. The sides of crucible were rinsed. This washing was performed twice. It was then continued by having the same washing twice with acetone. Stirring rod was also rinsed before removing. Crucible with its content of fibre residual was oven dried at 100^{0}C overnight, cooled in a desiccator and weighed. About 0.1 g of dried fibre residue was taken for N and C analysis using Elementar Vario Macro Cube analyzer as described previosusly in order to get NDFIP and NDFIC. Finally, the remaining residual fibre content was ashed at 550°C in furnace for 4.5 h, cooled in a desiccator and weighed.

Equation:

F : Wt. of tube (g)

FS: Wt. of tube with dried sample (g)

S : Wt. of dried sample (g); **S = FS - F**

C : Wt. of sintered glass crucible (g)

CR: Wt. of sintered glass crucible with dried fibre residue (g)

R : Wt. of dried fibre residue (g); **R = CR - C**

CA: Wt. of crucible with ash (g)

A : Wt. of ash (g) (after being corrected with the amount of fibre residue taken for N and C analysis)

N_p: N content in percent (%)

N: N in gram (g), **$N = N_p/100 \times 1000$**

$C_{p:}$ Carbon in percent (%), **$C = C_p \times 1000$**

$$\text{ADF (g/kg DM)} = \frac{R - A}{S} \times 1000$$

$$\text{ADFIP (g/kg DM Fibre)} = \frac{(6.25 \times \mathbf{N})}{S} \times \text{ADF}$$

$$\text{ADFIC (g/kg DM Fibre)} = \frac{\mathbf{C}}{S} \times \text{ADF}$$

2.2 Acid detergent lignin (ADL)

Apparatus:

1. See the apparatus used for ADF

Reagents

(a) Sulfuric acid (72 %) standardized to m.w. 1.64 (Added 420 ml H_2SO_4 (95-98% m.w. 1.834) to 580 ml H_2SO_4) in a 2 L volumetric flask put on ice in a fume cupboard

(b) Acetone

Procedure:

The initial procedure was similar to ADF determination in which after obtaining dried residual from the last step of ADF determination's procedure, sulfuric acid (72%) was added to about half full of crucible, stirred with glass rod and allowed it to drain (natural gravity filtration). The crucible was then refilled with the similar sulfuric acid, stirred hourly intervals for 3 times (3 h) and filtered with the aid of vacuum suction to fasten draining. Next, the residual content was washed with hot (90-100^{0}C) water until acid-free and rewashed again with acetone. The sides of crucible were rinsed and stirring rod removed after being rinsed. After this, the crucible and its content was dried at 100^{0}C in the oven overnight, cooled in a desiccator and weighed.

Finally, the residual content was ashed at 550°C in furnace for 4.5 h, cooled in a decicator and weighed.

Equation:

S : Wt. of dried sample (g) (obtained from ADF determination)

C : Wt. of sintered glass crucible (g) (obtained from ADF determination)

CR: Wt. of sintered glass crucible with dried residue (g)

R : Wt. of dried residue (g); **R = CR - C**

CA: Wt. of crucible with ash (g)

A : Wt. of ash (g)

$$\text{ADL (g/kg DM)} = \frac{R - A}{S} \times 1000$$

2.3 Total phenols and total tannins

These measurements were based on Folin-Ciocalteu method with using tannic acid as equivalent standard as described by (Makkar, 2003b)

Apparatus:

1. 20 ml and 10 ml test tubes
2. Gilson pipettes (0.02 ml, 0.1 ml, 1 ml, and 5 ml) (Gilson Inc, USA)
3. Vortex (whirly) mixer (Nikel Elector, UK)
4. Ultrasonic waterbath (Fisher scientific, UK)
5. Refrigerated centrifuge (Baird & Tatlock Ltd., UK)
6. Plastic UV cuvette
7. Spectrophotometre (Libra S12, Biochrom, UK)

Reagents:

(a) 70 % aquaeous acetone (v/v)

(b) An ultrasonic water bath (Fisher scientific, UK)

(c) Folin-Ciocalteu reagent (1N). Commercial Folin-Ciocalteu reagent (2N) (Fisher Scientific, UK) was equally diluted with distilled water, kept in a brown bottle and stored in cold room (4^oC). The colour should not be olive green.

(d) Sodium carbonate (20%). 40 g Sodium bicarbonate decahydrate (x10 H_2O) was dissolved in 200 ml of distilled water.

(e) (insoluble) Polyvinyl polypyrrolidone (PVPP) (Sigma, Germany)

(f) Standard tannic acid solution (0.1 mg/ml). 25 mg tannic acid (Fisher scientific, UK) was dissolved in 250 ml of distilled water (1:10). Fresh solution should be always used.

(g) Adjusted distilled water with pH 3. This was obtained by slowly adding very small amount of HCL into distilled water until the pH 3 reached.

Procedure:

Standard calibration

Initially, calibration of the standard was prepared by analyzing standard tannic acid solution up to 3 times and the tabulated results described as follow:

Table 3.1. Calibration standard of tannic acid

Tubes	Tannic acid solution (0.1 mg/ml) (ml)	Distilled water (ml)	Folin-Ciocalteu reagent (ml)	Sodium carbonate solution (ml)	Absorbance at 725 nm	Tannic acid (mg)
To	0.00	0.50	0.25	1.25	0.000	0.000
T1	0.04	0.46	0.25	1.25	0.193	0.004
T2	0.08	0.42	0.25	1.25	0.365	0.008
T3	0.12	0.38	0.25	1.25	0.557	0.012
T4	0.16	0.34	0.25	1.25	0.713	0.016

Regression equation (r^2 = 0.998) of tannic acid standard (mg): $(0.0223 \times absorbance\ at\ 725\ nm) - 0.000160$

Extract preparation

About 200 mg each of dried ground sample was put into a tube of about 20 ml capacity and 10 ml acetone (70%) added. After that, the tubes were then suspended in an ultrasonic water bath (without heating) and subjected to ultrasonic treatment for 2x10 minutes with 5 minutes break in between. The content of the tube was centrifuged using refrigerated centrifuge set at 4 ^{o}C at 3000 rpm for 10 minutes and the supernatant collected for the analyses.

Total phenols analysis

About 0.02 ml of each tannin-containing sample extract was transferred to the test tube of around 10 ml capacity and 0.48 distilled water added to make the volume up to 0.5 ml. It was then to add 0.25 ml of Folin-Ciocalteu reagent and 1.25 ml of sodium carbonate solution into the tube respectively. After that, the tube was vortexed, kept on the rack for 40 minutes and adequate solution in the tube transferred into cuvettes (usually in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 725 nm recorded against blank solution (T0). Total phenols (tannic acid equivalent) was calculated from the above calibration standard and if the value of absorbance reached higher than the range of calibration standard, the extract sample should be appropriately diluted.

Equation:

A: mg tannic acid

$$A = (0.0223 \times absorbance\ at\ 725\ nm) - 0.00016$$

B: mg tannic acid in 1 ml extract sample

$$B = \frac{A}{0.02}$$

C: As 200 mg dried ground sample was extracted in 10 ml solvent, it was equivalent to 100 mg dried ground sample was extracted in 5 ml solvent.

Thus, 100 mg dried ground sample = $5 \times$ B mg tannic acid (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g tannic acid

$$C = 5 \times (B \times 10) \text{ g tannic acid}$$

Total phenols (g/kg DM tannic acid equivalent) = $\frac{C \times dilution\ factor}{kg\ DM} \times 1000$

If the extract sample was not diluted, the dilution factor should be 1 (one).

Total tannins analysis

In this procedure, PVPP (a tannins binding agent) was used in order to remove tannins form extract sample. About 100 mg PVPP was put into a test tube (10 ml capacity) and 1 ml of adjusted distilled water (pH 3) as well as 1 ml of each extract sample added respectively. It was then to vortex the tubes and to keep them in cold room (4^{o}C) for 15 minutes. Next, each tube was vortex and subjected to refrigerated centrifugation (at 3000 rpm and 4^{o}C) for 10 minutes. After that, supernatant was collected and subjected to total phenols analysis. This supernatant had only simple phenols other than tannins since it had been precipitated along with PVPP. About 0.1 or 2.0 ml of supernatant was transferred to the test tube of around 10 ml capacity and 0.4 distilled water added to make the volume up to 0.5 ml. It was then to add 0.25 ml of Folin-Ciocalteu reagent and 1.25 ml of sodium carbonate solution into the tube respectively. After that, the tube was vortexed, kept on the rack for 40 minutes and adequate solution in the tube transferred into cuvettes (usually in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 725 nm recorded against blank solution (T0). Total simple phenols (tannic acid equivalent) was calculated from the previous calibration standard and if the value of absorbance reached higher than the range of calibration standard, the extract sample should be appropriately diluted.

Equation

A: mg tannic acid

$$A = (0.0223 \times absorbance\ at\ 725\ nm) - 0.00016$$

B: mg tannic acid in 1 ml extract sample

$$B = \frac{A}{0.1 \text{ or } 0.2}$$

C: As 200 mg dried ground sample was extracted in 10 ml solvent, it was equivalent to 100 mg dried ground sample was extracted in 5 ml solvent.

Thus, 100 mg dried ground sample = $5 \times$ B mg tannic acid (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g tannic acid

Due to equal dilution of extract sample with adjusted distilled water (pH3) (1 ml extract sample : 1 ml adjusted distilled water pH 3) during tannins removal by PVPP

Therefore, 1 kg dried ground sample = $(5 \times 2) \times (B \times 10)$ g tannic acid

$C = 10 \times (B \times 10)$ g tannic acid

$$\text{Total simple phenols (g/kg DM tannic acid equivalent)} = \frac{C \times dilution\ factor}{kg\ DM} \times 1000$$

Total tannins (g/kg DM tannic acid equivalent) = total phenols - total simple phenols

2.3 Condensed tannins

This procedure was basically referred to Porter et al. (1986) as described by (Makkar, 2003b) with using (-)- epigallocatechin gallate (Sigma, UK) as standard equivalency. This particular catechin is known to be the most abundant one in green tea.

Apparatus:

1. 20 ml test tubes with loose lids
2. Gilson pipettes (0.02 ml, 0.1 ml, 1 ml, and 5 ml) (Gilson Inc, USA)
3. Vortex (whirly) mixer (Nikel Elector, UK)
4. Ultrasonic waterbath (Fisher scientific, UK)
5. Refrigerated centrifuge (Baird & Tatlock Ltd., UK)
6. Heating mantle (Barnstead electrothermal, UK)
7. 100 ml flasks (Quickfit, UK)
8. Plastic UV cuvette
9. Spectrophotometre (Libra S12, Biochrom, UK)

Reagents:

(a) 70% aqueous acetone (v/v)

(b) Standard solution of (-)- epigallocatechin gallate (Sigma, UK). 2 mg (-)-epigallocatechin was dissolved in 2 ml 70% aqueous acetone (v/v) (1mg : 1 ml)

(c) Butanol-HCL reagent (butanol-HCL 95:5 v/v): 950 ml n-butanol and 50 ml HCL (36-37%) were mixed

(d) Ferric reagent (2% ferric ammonium sulfate in 2 N HCL): 16.6 ml of HCL (36-37%) was transferred into a 100 volumetric flask and distilled water added to make the volume up to 100 ml (2 N HCL). After that, 2 g ferric ammonium sulfate was dissolved into it. The final reagent was then stored in a dark bottle.

Procedure:

Standard calibration

Initially, calibration of the standard was prepared by analyzing (-)-epigallocatechin gallate standard solution up to 3 times and the tabulated results described as follow:

Table 3.2. Calibration standard of (-)-epigallocatechin gallate

Tubes	(-)-epigallocatechin solution (1mg/ml) (ml)	Acetone 70% (v/v) (ml)	Butanol-HCL reagent (ml)	Ferric reagent (ml)	Absorbance at 550 nm	(-)-epigallocatechin (mg)
To	0.00	0.50	3.0	0.1	0.000	0.00
T1	0.10	0.40	3.0	0.1	0.052	0.10
T2	0.20	0.30	3.0	0.1	0.116	0.20
T3	0.30	0.20	3.0	0.1	0.166	0.30
T4	0.40	0.10	3.0	0.1	0.215	0.40
T5	0.50	0.00	3.0	0.1	0.285	0.50

Regression equation (r^2 = 0.997) of (-)-epigallocatechin gallate (mg): $(0.00331 + 1.78\ X\ absorbance\ at\ 550\ nm)$

Extract preparation

A 100 mg each of dried ground sample was put into a tube of about 20 ml capacity and 5 ml acetone (70%) added. After that, the tubes were then suspended in an ultrasonic water bath (without heating) and subjected to ultrasonic treatment for 2x10 minutes with 5 minutes break in between. The content of the tube was centrifuged using refrigerated centrifuge set at 4 ^{o}C at 3000 rpm for 10 minutes and the supernatant collected for the analyses.

Condensed tannins analysis

A 0.1 ml each of sample extract was transferred in to a tube of about 20 ml capacity and 0.4 70% aqueous acetone added to make the volume up to 0.5 ml. Next, 3.0 ml of butanol-HCL reagent and 0.1 ml ferric reagent were added respectively. The tube was then vortexed and loosely closed with a lid before putting it in boiling water (around 100^{o}C) for 60 minutes.

Boiling water was obtained by heating flask with water in it using heating mantle. After that, the tube was cooled in cool water for 3-5 minutes, vortexed and adequate solution transferred into cuvettes (in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 550 nm recorded against a suitable blank solution. As all the sample extracts had considerable amount of flavan-4-ols, a pink colour developed just after heating especially for the original green tea leaves (GTL), each suitable blank solution has been made from another similar solution for each sample but being subjected to heating only 10 to 15 minutes. This range of time was chosen because when the standard of (-)- epigallocatechin gallate had been made, the colour changes of the solution during heating was appeared usually after 20 minutes.

Equation:

A: mg (-)-epigallocatechin gallate

$$A = (0.00331 + (1.78\ X\ absorbance\ at\ 550)$$

B: mg (-)-epigallocatechin gallate in 1 ml extract sample

$$B = \frac{A}{0.1}$$

C: As 100 mg dried ground sample was extracted in 5 ml solvent,

Thus, 100 mg dried ground sample = $5 \times$ B mg (-)-epigallocatechin gallate (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g (-)-epigallocatechin gallate

C = $5 \times (B \times 10)$ g (-)-epigallocatechin gallate

Condensed tannins (g/kg DM (-)-epigallocatechin gallate equivalent = $\frac{C}{kg\ DM} \times 1000$

2.3 Determination of antioxidant capacity: this was examined using FRAP method according to the procedure of Benzie and Strain (1996).

Apparatus

1. Weighing scale
2. Centrifuge
3. Sterile graduated pipettes
4. Centrifuge tubes
5. Gilson pipette and tips
6. UV/ Spectrophotometer
7. Water bath
8. Disposable plastic cuvette

Reagents

(a) 2, 4, 6,-tri (2-pyridyl)-s-triazine (TPTZ) Sigma Aldrich
(b) Iron (III) chloride 6- hydrate
(c) Iron (II) sulphate 7- hydrate
(d) Glacial acetic acid
(e) Sodium acetate trihydrate
(f) Hydrochloric acid
(g) Methanol

Sample Preparation

About 0.5g of the dried forage sample (1mm in size) was measured into a boiling test tube and 10ml of methanol- water (8:2, v/v) was added to it. This was kept in a shaking water bath at 35^0C for 24hours as described by Cai *et al.*, (2004). The mixture was allowed to cool at room temperature and subsequently centrifuged at 4000rpm for 10mins at 4^0C. The supernatant was obtained and used for antioxidant capacity determination of the samples.

FRAP standard preparation

Ferrous sulphate was used in preparing standard solutions with a wide range of concentration to suit the samples under investigation.

Ferrous sulphate (FeSO4.7H20): 0.278g of ferrous sulphate was dissolved in 500ml of distilled water to give 2mM or 2000µM of ferrous sulphate

FRAP Chemicals needed for FRAP solution

1. **Acetate buffer (300mM, pH 3.6)**: 3.1g of sodium acetate trihydrate, 16ml of glacial acetic acid, distilled water to 1 litre, check the pH and store at 4^0C.
2. **Dilute HCl (40mM):** 1.46ml conc. HCl (32-36%) 11M, distilled water to 1 litre and store at room temperature
3. **TPTZ (10mM)**: 0.031g TPTZ in 10ml of 40mM HCl, dissolved at 50^0C in water bath (It was prepared on the day of assay in a corning centrifuge tube)
4. **Ferric chloride (20mM):** 0.054g of iron (III) chloride 6- hydrate dissolve in 10ml distilled water (Always prepare fresh solution on the day of assay in a corning centrifuge tube).

FRAP solution preparation

Firstly sodium acetate buffer (300mM, pH 3.6), 10mM TPTZ solution (40mM HCl) and 20mM ferric chloride were mixed at a volume ratio of 10:1:1 to generate the FRAP reaction solution

which was freshly prepared on the day of assay. This was warmed to 37^0C in a water bath before use.

Standard Stock ferrous sulphate was diluted as given in the range of standards as shown below:

Standard	Concentration (mM)	Ferrous sulphate (2mM)	Water
1	0	0	1
2	0.2	0.1	0.9
3	0.4	0.2	0.8
4	0.8	0.4	0.6
5	1.2	0.6	0.4
6	1.6	0.8	0.2
7	2.0	1.0	0

Standard / sample composition: 0.01ml of each concentration of the standard and or sample solution were mixed separately with 0.2 ml of the FRAP solution. Standard curve was prepared on daily basis for each assay, the slope of the standard curve was calculated and this was used to convert the absorbance values of the forage samples to an equivalent ferrous sulphate concentration. After 4mins of reaction, the absorbance of the mixtures (i.e standard and sample) were recorded at 593nm (Wong *et al*., 2006) using microplate reader. The results obtained were expressed as μmol Fe (II) g/dry weight of the forage sample.

2.4 IVDMD and IVOMD calculations

IVDMD

A: Wt. of crucible (g)

B: Wt. of fresh sample before incubation (g)

C: Dry weight of sample used for incubation = B (g) x DM content obtained from chemical analyses (g)

D: Wt. of crucible with dried residue after incubation (g)

E : dry weight of residue after incubation (g) **= D – A**

F: Degraded dry matter (g) = C (g) – E (g)

$$\text{IVDMD (g / kg)} = \frac{\text{F (g)}}{\text{C}} \times 1000$$

IVOMD

A: Wt. of crucible (g)

B: Wt. of crucible with dried residue after incubation (g)

C: Wt. of dried residue after incubation (g) from IVDMD; B-A

D: Wt. of crucible with ash after incubation (g)

E: Wt. of ash (g) = $CS_1 - A$

F: OM content of the incubated sample = C (g) x OM content obtained from chemical analyses (g)

G: Un-degraded OM after incubation (g) **= C – E**

DOM: Degraded Organic matter (g) = F (g) – G (g)

$$\text{IVOMD (g / kg)} = \frac{\text{H (g)}}{\text{F}} \times 1000$$

2.5 Volatile fatty acids calculation

Acid	**Molecular weight (g/mol)**
Acetic acid	60.05
Propionic acid	74.08
n- Butyric acid	88.11
Isobutyric acid	88.11
n-Valeric acid	102.13
Isovaleric	102.13
Formate	46.03

Note: mg/l = mg/kg = ppm

Example: calculate the acetate in sample C3: $\text{mmol/L} = \frac{\text{ppm or mg / L or mg / kg}}{\text{molecular weight of acetate}}$

$$\text{mmol/L} = \frac{286.140}{60.05} = 4.765$$

The rumen fluid and forage extract were mixed with 0.4ml of metaphosphoric acid (i.e. 2ml in 2.4ml)

$$\text{Acetate in forage extract} = \frac{4.765 \text{ x } 2.4}{2.0} = 5.718 \text{ mmol / L.}$$

Note: the dilution factor for the OSA has already been incorporated in the analysed result obtained from the machine

3.3.3.4.1 Acetate: Propionate (A: P) calculation.

$$\frac{Acetic\ acid\ (\frac{mmol}{L})}{Propionic\ acid\ (\frac{mmol}{L})}$$

Appendix 3.1: Total bacterial counts, using SYBR gold nucleic acid stain / Acridine orange (Tuma *et al.*, 1999).

Sample preparation

1) **Apparatus and Reagents for total Bacterial Count**

A) Apparatus

- Microscope, vertical UV illuminator for epifluorescence with flat field 100x oil immersion objective lens, to give total magnification of at least 1000x
- Counting graticule, ocular lens micrometre calibrated with stage micrometre.
- Filters, including excitation filters (KP 490 and LP 455) beam splitter (LP 510) and barrier filter (LP 520 using mercury lamp, HBO 50).
- Blender or vortex mixer.
- Filtration unit, suitable for use with 13mm diameter membrane filters.
- Membrane filters, polycarbonate: 13mm diameter, 0.2µm pore size (non-fluorescent); cellulosic 25mm diameter, 5µm pore size.
- Syringes, 3ml, disposable syringe filters, 0.2 µm pore size.
- Test tubes, glass, screw-capped, 13 x 125 mm.
- Necessary PPE.
- Beakers.

- Forceps.
- Air drier

B) Reagents

- Phosphate buffer (Oxoid Phosphate buffered saline tablets (Dulbecco A), BR0014): dissolve 20 tablets / litre (2X PBS) of distilled water, it was then autoclaved for 10 mins at 115^0C
- Fixative 4% (w/v) Paraformaldehyde in phosphate buffer: For 1 L of 4% Formaldehyde, 800 mL of 1X PBS was added to a glass beaker on a stir plate in a ventilated hood. This was heated to approximately 60 °C on a hot plate while stirring. It was ensured that the solution didn't boil over from the beaker. About 40 g of paraformaldehyde powder was then added to the heated PBS solution. 1 N NaOH was then added to the solution drop wisely from a pipette until the solution clears since the powder did not dissolve immediately in the solution. Once the paraformaldehyde was found to have dissolved completely, the solution was cooled and then filtered through 0.4 µm filter. The volume of the solution was then made up to 1 L with 1X PBS. The pH was then rechecked and adjusted with small amounts of dilute HCl (1M) to approximately 6.9. The solution was then aliquoted and frozen
- Fixative 2% (w/v) Paraformaldehyde in phosphate buffer: half of the 4% PFA prepared was diluted with the same amount of 1 x PBS, the pH was checked and adjusted with small amounts of dilute HCl (1M) to approximately 6.9. The solution was then aliquoted and frozen
- Fluorochrome (SYBR gold 100X): this was prepared by adding 10 µl of the 10000X SYBR gold to 990 µl of filtered 1 X PBS.
- Immersion oil, low fluorescing.

Procedure

1) Bacterial cells were diluted to a suitable dilution (10^9) in filter sterile 1X PBS. In order to get cell counts between 30-300 cells per field of view.

2)

i) SYBR gold: About 50 µl of SYBR gold (diluted 100X in filter sterile PBS) was then added to each 1ml of sample.

ii) Acridine orange: About 1 ml of Acridine orange was then added to each 1ml sample

3) SYBR gold is light sensitive, so the mixture was wrapped with foil and incubated at room temperature for 30mins

4) Controls:

i) SYBR gold: 1 X PBS control – 1 ml of autoclaved PBS was added to 50 µl SYBR gold (100 X diluted), wrap in foil and incubate for 30 min.

ii) Acridine orange: 1 X PBS control – 1 ml of autoclaved PBS was added to 1 ml of Acridine orange (0.1% w/v).

5) Control measures

i) Non-SYBR gold stained cells was also used as a control measure.

ii) Non- acridine orange cells was also used as a control measure.

Filtration

1) Sterile Millipore filter holder was autoclaved to maintain sterility.

2) Filter holder was then unscrewed and a single 13mm membrane filter (shiny side facing up) was then transferred aseptically with forceps to the base of filter cradle and then the filter cradle was re-assembled.

3) The filter holder was then sit on the bung of volumetric flask which was attached to a vacuum pump.

4) The vacuum pump was then switched on and the whole sample was pipette into the filter holder after giving it a shake.

5) The sample was allowed to be drawn onto the filter after which it was washed through with 3 x 1 ml filter sterile water. After all the solutions have been drawn through the filter the vacuum pump was left running to dry filter for 2-3 min.

6) Small drop of Citifluor (stored at 4°C) was placed onto a clean microscope slide.

7) The filter holder was unscrewed, and with forceps, the dried membrane filter was aseptically transferred onto the drop of Citifluor.

8) Another small drop of Citifluor was placed on top of the membrane and coverslip was finally placed over the membrane filter.

9) The slide was labelled and nail varnish was used to seal the coverslip unto the slides.

10) The prepared slide was then placed in the dark to stop fluorescence from fading.

11) The slides were viewed using 100x oil immersion (UPlanFl 100x lens), under blue light filter (WB) in the dark.

11) The image was focussed down the microscope lens, and the picture was taken.

Calculating cells per ml of original sample

1) Calculate average number of cells per field of view, with error

2) Calculate no. of fields of view on the filter membrane e.g.

Diameter of membrane = 9.5 mm, Radius of membrane (r) = 4.75 mm

Therefore, area of membrane = πr^2 = 70.88 mm^2

(NB: each graduation on the stage micrometer is 10 μM)

Area of field of view (calculated using image of stage micrometer) = 100 μM x 120 μM = 0.1 mm x 0.12 mm = 0.012

Area of membrane 70.88

Area of fields of view 0.012 = 5906.667

3) Multiply average number of cells per field of view by 5906.667

4) Multiply result of 3) by dilution factor (e.g. 200X, 100X, 20X)

5) Multiply result of 4) by original sample dilution factor (e.g. if samples were originally stored in a 50/50 mix of sample and PFA – multiply by 2).

6) Result is cells per ml of original sample.

Appendix 3.2: Total fungal counts, using Calcofluor white (Harrington and Hageage, 2003).

1) **Apparatus and Reagents for total fungal count**

A) Apparatus: it requires the sae apparatus as that of bacteria it is only the membrane filter that was different

- Membrane filters, polycarbonate: 13mm diameter, 0.6μm pore size.

B) Reagents

- Phosphate buffer (Oxoid Phosphate buffered saline tablets (Dulbecco A), BR0014): dissolve 10 tablets / litre (1X PBS) of distilled water, it was then autoclaved for 10 mins at 115^0C
- **10% KOH**: 11.77 g potassium hydroxide was dissolved in 20 mL glycerol, then 80 mL distilled water was added to it; this was stored store at 25 °C.
- Fixative 4% (w/v) Paraformaldehyde in phosphate buffer: as described in Appendix 1
- Fluorochrome: Calcofluor white (0.1% (w/v) in 10% KOH)
- Immersion oil, low fluorescing.

About 1ml of the sample was added to glass, screw-capped test tube (13 x 125mm) containing 9ml fixative (4% formaldehyde in PBS), after fixing of samples, samples were stored at 4^0C for up to 3 weeks without significant decrease in cell numbers. Further dilutions of samples was done if it is difficult to obtain reproducible results. This was done by mixing sample using a vortex mixer from which ten-fold dilutions in phosphate buffer solution was prepared.

One ml of the sample or diluted sample was placed on a non-fluorescent polycarbonate filter supported by a cellulosic membrane filter in a filter holder and 1 ml of calcoflour white was added to it using a disposable sterile syringe filters (3ml capacity; 0.6 µm pore size). This was left for 10min after which it was washed thoroughly with autoclaved PBS solution by using gentle vacuum (about 20KPa) for 2-3 times with the PBS used for washing being about 4 times the volume of the fixed sample. The polycarbonate filter was allowed to dry in the membrane filter for about 1 – 2 min. The membrane filter was removed aseptically with a forceps onto a clean microscope slide containing a drop of non-fluorescent immersion oil (Cargille type A). A

drop of immersion oil was added to the filtrate surface and it was gently covered with a clean glass cover slip, this can be stored in this condition for several months without significant fluorescence loss.

Calculations: it followed the same principle of the bacterial count